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(54) Title: DNA PROMOTER SEQUENCES DERIVED FROM BANANA BUNCHY TOP VIRUS

(57) Abstract

This invention relates to DNA promoter sequences derived from components of banana bunchy top virus. In one aspect, the invention is a DNA molecule including a promoter sequence upstream of a cloned gene and adapted for expression in a plant cell. In a furtheraspect, the invention is a DNA molecule including a promoter sequence and an intron upstream of a cloned gene. The invention also relates to the expression of cloned genes with the DNA molecule including a promoter sequence from a banana bunchy top virus component and an intron upstream of a cloned gene enabling expression in monocotyledon and/or dicotyledon plant cells and plants.

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DNA PROMOTER SEQUENCES DERIVED FROM BANANA BUNCHY TOP VIRUS

Field of Invention

This invention relates to DNA promoter sequences. In particular this invention relates to DNA sequences derived from Banana Bunchy Top Virus for promoting transcription of an cloned gene or gene fragment. The invention also relates to DNA constructs encoding these promoter sequences and hosts such as bacterial and plant cells harbouring these DNA constructs.

Background Art

With the development of more efficient and reliable systems for plant transformation and regeneration, interest in the generation of transgenic plants with new traits such as disease, insect and drought resistance has grown. Transgene expression is an essential component in the development of transgenic plants and the promoter driving the transgene is of major importance (Mitra. A. and Higgins, D (1994) Plant molecular Biology 26:85-93; McElroy et al. (1991) Molecular and General Genetics 231:150-160; Last et al. (1993) Theoretical and Applied Genetics 81:581-488).

The most commonly used plant promoter for transgene expression is the cauliflower mosaic virus 35S (CaMV 35S) promoter (Guilley et al.(1982) Cell 30:763-773). The CaMV 35S promoter is considered a strong promoter and has been used for the expression of foreign genes in both dicots and monocots. However, the activity of the CaMV 35S promoter in monocots is much lower relative to dicots (Last et al. (1991) supra;

McElroy et al (1991) supra; Schledzewski and Mendel (1994). Transgenic Research 3:249-255; Ralthus et al. (1993) Plant Molecular Biology 23:613-618). This has become a major limitation in transformation of monocot species particularly within the Graminaceae (McElroy et al. (1991) supra). Thus, a number of monocot promoters have been isolated and characterised from both monocots and monocot infecting viruses.

The most commonly used plant-derived monocot promoters include the maize alcohol dehydrogenase (adh1) promoter (Dennis et al. (1984) Nucleic Acids Research 12:3983-4000), the rice actin promoter (act1) (McElroy et al. (1991) supra), the maize polyubiquitin promoter (ubi1) (Christensen et al. (1992) Plant Molecular Biology 18:675-689), and the recombinant promoter, pEMU (Last et al. (1991) supra). All these promoters appear to be constitutive, but differ in their level of activity between monocot species (Wilmink et al. (1995) Plant Molecular Biology 28:949-955).

Promoters derived from monocot viruses have generally been tissue specific. Viral promoters derived from badnviruses (rice tungro bacilliform virus (RTBV) and commelina yellow mottle virus (CoMYV) (Bhattacharyya-Pakrasi et al. (1993) The Plant Journal 4:71-79; Yin and Beachy (1995) The Plant Journal 7:969-980; Medberry et al. (1992) Plant Cell 4:185-192; Medberry and Olszewski (1993) The Plant Journal 3:619-62) and the geminiviruses (maize streak virus (Fenoll et al. (1988) The Embo Journal 7:1589-1596; Fenoll et al. (1990) Plant Molecular Biology 15:865-77) have been shown to be phloem specific.

Recently multicomponent circular ssDNA plant viruses including banana bunchy top virus (BBTV), coconut foliar decay virus (CFDV), subterranean clover stunt virus (SCSV) and faba bean necrotic yellows virus (FBNYV) have been identified (Burns et al. (1995) Journal of General Virology 76:1471-1482; Katul et al. (1995) Journal of General Virology 76:475-479; Rohde et al. (1990) Virology 176:648-651).

Thirteen different ssDNA components have been isolated from BBTV infected plants (Burns et al. (1995) Journal of General Virology 76:1471-1482; Yeh et al. (1994) Virology 198:645-652; Wu et al. (1994) Phytopathology 84:952-58; Wu (1994). Journal of Phytopathology 128:153-160). Each component is approximately 1 kb and contains one major open reading frame (ORF) in the virion sense. Of the thirteen components only six (BBTV DNA-1 to 6) appear to occur in all infections tested and thus are considered to be the essential components of the BBTV genome (Burns et al. (1995) supra; Harding et al.(1993) J Gen Virol 74: 323-328).

Each of these six components has been shown to encode at least one gene through RNA transcript analysis (Beetham et al.(1997) J Gen Virol 78: 229-236). BBTV DNA component 1 (BBTV DNA-1) contains two ORFs in the virion sense. The major ORF encodes a replication (Rep) protein (Harding et al. (1993) supra). The functions of the genes encoded by BBTV DNA-2, -4, -5 and -6 are not known. The nucleotide sequence of these components have been determined (Karan (1995) Ph.D. thesis, Queensland University of Technology).

The other seven components have been found with only some BBTV isolates. Each of these additional components is similar to BBTV DNA-1 in that they potentially encode a replication protein (Rep) based on the presence of a dNTP-binding motif and rolling circle replication motifs. The nucleotide sequence of two of the additional components BBTV-S1 and S2 has been determined. The nucleotide sequence of their replication proteins shows low sequence homology to the nucleotide sequence of the replication proteins of BBTV DNA-1 compared with the higher sequence homology to the nucleotide sequence of the replication proteins of SCSV, FBNYV and CFDV (Karan (1995) Ph.D. thesis, Queensland University of Technology).

Summary of the Invention

It is an object of the present invention to provide a DNA molecule including a promoter sequence derived from BBTV component 6, S1 or S2 for promoting transcription of a cloned gene in a plant cell.

In one aspect the invention resides broadly in an isolated DNA molecule including a promoter sequence derived from a substantially untranslated portion of any one BBTV component and adaptable for promoting transcription of a cloned gene in a plant cell.

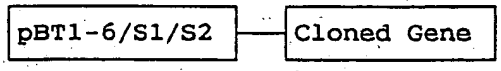
Preferably the promoter sequence is derived from any one of BBTV components 1-6 (BBTV1-6), S1 (BBTVS1), or S2 (BBTVS2).

Preferably the promoter sequence is derived from the intergenic region of the above mentioned BBTV components.

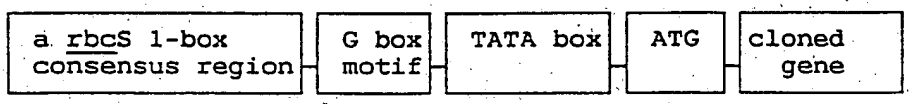
In one preferred embodiment the promoter sequence is derived from the intergenic region of BBTV component 6. In another preferred embodiment the promoter sequence is derived from the intergenic region of BBTV components S1 or S2.

By way of example, the promoter sequences described above will be represented with reference to BBTV 1-6 (pBT1-6), BBTV S1 (pBTS1), or BBTV S2 (pBTS2).

The BBTV 1-6, S1, or S2 promoter sequences (pBT 1-6/S1/S2) and the cloned gene may be represented as follows:



Preferably pBT1-6/S1/S2 includes an ATG initiation codon, a TATA box (CTATTAATA), a G-box motif (CAGCTG), and a rbcS 1-box consensus region. The order of the specific DNA sequences is preferably as follows:



The pBT1-6/S1/S2 may include additional elements including an ocs-like 3' region, an ocs-like 5' region, a CR-SL region, a MSV rep-1-like motif, and or a CR-M region. These elements are preferably located upstream of the rbcS 1-box consensus region.

In one embodiment, the pBT6 includes pBT6.1, pBT6.2, pBT6.3, pBT6.4 or pBT6.5 as described herein.

The DNA sequence of the pBT1-6 region may be identical or substantially identical to the BT6 sequences described herein. Substantially is used in this specification to refer to sequences having variations up to 20%. The amount of sequence variation can be determined by standard hybridisation procedures or sequence comparison. The percentage of 20% is the variation shown with the region outside of the ORF of

component 1 between different geographical isolates (Karan et al, 1994, Journal of General Virology, 75, 3541-3546; and US Patent Application No 08/202,186). Both of these documents are herein incorporated by reference to support the claim of 20% variation of all components of BBTV. The variation determined for component 1 of different geographical isolates is representative of the variation between each component from different geographical isolates. Thus variation up to 20% equally applies to the sequences of all components.

The term derived defines any sequence that has been changed, altered or modified by whatever procedure including mutagenesis from a fragment of the BBTV component 1-6.

The DNA molecule may include one or more introns between the promoter and the cloned gene. The intron is preferably an intron located substantially upstream of a translated region of a gene but downstream of pBT1-6. Preferably, the intron is the first intron of a 5' untranslated region (UTR). The intron is preferably the first intron of the UTR of maize alcohol dehydrogenase 1 (adh1), maize polyubiquitin 1 (ubi1) or rice actin. The inclusion of an intron to the DNA molecule may be represented as

Two or more introns may be inserted in tandem between pBT1-6 and the cloned gene.

The cloned gene may encode a full or partial structural protein.

The gene of interest may be any suitable gene such as GUS, NPTII, insecticide resistance gene, herbicide resistance gene or a growth promoting gene.

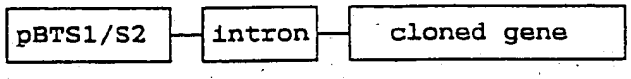
The plant cell may be a monocotyledon plant cell such as a banana cell or dicotyledon plant cell such as a tobacco cell.

In another embodiment, pBTS1 is S1-L, S1-I or S1-P and pBTS2 is S2-I or S2-P as described herein.

The DNA sequence of the pBTS1/S2 region may be identical or substantially identical to the BBTV S1 or S2 sequences described herein. Substantially is used in this specification to refer to sequences having variations up to 20%. The amount of sequence variation can be determined by standard hybridisation procedures or sequence comparison. The percentage of 20% is the variation shown with the region outside of the ORF of component 1 between different geographical isolates (Karan et al. (1994) Journal of General Virology, 75, 3541-3546; and US Patent Application No 08/202,186). Both of these documents are herein incorporated by reference to support the claim of 20% variation of all components of BBTV. The variation determined for component 1 of different geographical isolates is representative of the variation between each component from different geographical isolates. Thus variation up to 20% equally applies to the sequences of all components.

The term derived defines any sequence that has been changed, altered or modified by whatever procedure including mutagenesis from a fragment of the BBTV component S1 or S2.

The DNA molecule may include one or more introns between the promoter and the cloned gene so as to increase expression of the cloned gene. The intron is preferably an intron located substantially upstream of a translated region of the gene but downstream of pBTS1/S2. Preferably, the intron is the first intron of a 5' untranslated region (UTR). The intron is preferably the first intron of the UTR of maize alcohol dehydrogenase 1 (adh1), maize polyubiquitin 1 (ubi1) or rice actin 1 (act1). The inclusion of an intron to the DNA molecule may be represented as



Two or more introns may be inserted in tandem between pBTS1/S2 and the cloned gene.

The cloned gene may encode a full or partial structural protein.

The cloned gene may be any suitable gene such as GUS, NPTII, a gene conferring insecticide resistance, a gene conferring herbicide resistance or a growth promoting gene.

The plant cell may be a cell from maize, banana, corn, wheat, rice, sugar cane, cuccumber, and tobacco.

The plant cell may be a monocotyledon plant cell such as a banana cell or dicotyledon plant cell such as a tobacco cell.

The invention in a second aspect is a plant cell having a DNA molecule as described above.

The invention in a third aspect is a plant with the plant cells as described above. The plant may be maize, banana, corn, wheat, rice, sugar cane, cuccumber, and tobacco.

The invention in a fourth aspect provides a method of expressing a gene in a plant cell using the DNA molecule as described above including

transforming the DNA molecule in a plant cell;

culturing the plant cell with the transformed DNA molecule; and

expressing the cloned gene in the plant cell with the transformed DNA molecule.

The invention will now be described with reference to preferred embodiments. These preferred embodiments are given by way of example only.

Brief Description of the Drawings

The preferred embodiments of the invention are described with reference to the accompanying drawings in which:

Fig 1 shows a nucleotide sequence of the BBTV DNA-6 intergenic region. The translational start and stop codons of the major virion sense ORF are in bold. The A in the ATG start codon of the major virion sense ORF is designated nucleotide position +1. Consensus TATA box, common region-major (CR-M), and common region-stem/loop (CR-SL) are shaded. Sequence motifs with homology to characterised cis-elements are boxed (motifs with similarities to the MSV rep-1 element, 5' and 3' regions of the

20 bp ocs-consensus, I-box element of rbcS promoters, and related G-box motif are indicated). Primers 1-4 and restriction sites AccI and HaeIII used to generate promoter fragments BT6.1-BT6.5 are also indicated.

Fig 2 shows a construction of BT6-uidA fusions. Promoter fragment BT6.1 (623 bp) was PCR amplified using primers 1 and 2. A unique AccI restriction site (nt-353 to -348) was used to remove a 272 bp region and generate the promoter fragment BT6.2 (351 bp). Promoter BT6.3 (239 bp) was PCR amplified using primers 1 and 3. A 3' deletion removing 147 nt was PCR amplified using primers 1 and 4 to generate promoter fragment BT6.5 (476 bp). Each promoter fragment was cloned upstream of the uidA reporter gene in pBI101.3 for Agrobacterium-mediated transformation of tobacco. The BT6-uidA-nos cassettes from each construction were inserted into pGEMzf$^+$ as HindII/EcoRI fragments for micro-particle bombardment.

Fig 3 shows a transient expressin of BT6 promoter fragments in NT-1 cells. BT6-uidA fusions were introduced into NT-1 cells via micro-particle bombardment. Promoter activity is expressed as pmol MU/min/mg total protein. Bars represent the mean ± standard errors of three independent experiments in which each promoter fusion was bombarded four times. pGEM35S-GN is the positive control in which the 800 bp CaMV 35S promoter drives uidA expression. The untreated column represents background GUS activity from unshot NT-1 cells.

Fig 4 shows a distribution of NPTII in leaves and roots of transgenic tobacco. Levels of NPTII are given as ng NPTII/mg total protein. Two month old, R_0 transformants, harbouring either BT6.3-NPTII (solid), nos-NPTII (cross-hatch), or CaMV 35S-NPTII (shaded) fusions were assayed for NPTII levels in newest leaves and roots via NPTII ELISA. Eight lines of BT6.3-NPTII transformed tobacco were compared to three lines of tobacco transformed with either nos-NPTII or 35S-NPTII fusions. To compensate for the high levels of NPTII detected in the 35S-NPTII transformed tobacco the y-axis has been broken. The

untransformed columns (open) represent background NPTII levels in three wildtype tobacco plants.

Fig 5 shows a transient expression of BT6 promoters in banana embryogenic cells. (a) Promoters including BT6.1, maize ubi1, maise adh1,. and CaMV 35S (530bp), fused to uidA, were introduced into banana embryogenic cells via micro-particle bombardment. Promoter activity is expressed as pmol MU/min/mg total protein. Figures represent mean activities ± standard errors for three independent experiments in which each promoter fusion was bombarded five times. (b) Promoters BT6.1, maize ubi1 and CaMV35S fused to a synthetic GFP reporter gene were introduced into banana cells via micro-particle bombardment. Promoter activity was estimated as the number of green fluorescent foci observed within a 40mm^2 region on the petri dish at a magnification of x13. Figures represent mean number of fluorescent foci ± standard error for three independent experiments in which each promoter fusion was bombarded six times. Superscript letters indicate a significant difference at 95% CI by one way analysis of variance.

Fig 6 shows a transient promoter activity in banana cv. Bluggoe embryogenic cells. Figures represent mean GUS activities ± standard error for three independent experiments.

Fig 7 shows a transient promoter activity in banana embryogenic cells as determined using the GFP reporter. Figures represent mean number of green fluorescent foci ± standard error for two independent experiments.

Fig 8 shows a nucleotide sequence of the BT6 terminator. Bold letters represent sequence homologous to primers BT6.ter and BT.HOMO.COM.

Fig 9 shows maize polyubiquitin (ubi-1) intron constructions.

Fig 10 shows maize alcohol dehydrogenase (adh-1) intron constructions.

Fig 11 shows rice actin (act) intron constructions.

Fig 12 shows sugarcane rubisco (rbcS) intron constructions.

Fig 13 shows multiple intron constructions.

Fig 14 shows transient expression studies with the six promoter sequences derived from BBTV-S1 and S2 in NT cells. Each was replicated three times and the data represents the combined results. The dicot CaMV 35S promoter from pBI121 was used as a control (35S). WT represents unbombarded tissue assayed identically to the bombarded tissue. The CaMV 35S promoter was given an activity of "1", and the relative strengths of the other promoters measured from this. Error bars show the 95% confidence intervals on the means.

Fig 15 shows schematic diagrams of linearized BBTV-S1 and S2 components, from which the six promoter sequences were derived. The region between the ORF represents the intergenic region including the polyadenylation (poly A) A site, TATA box and stem-loop (SL).

Fig 16 shows histochemical staining of a transgenic tobacco plant transformed with the S1-L promoter driving GUS. In both the leaves (Fig 16a) and roots (Fig 16b) GUS expression can be visualized by a blue precipitate. Expression appears to be limited to the vascular tissue of the leaves and roots. The root hairs and meristem also expressed GUS.

Fig 17 shows histochemical staining of a transgenic tobacco plant transformed with the S2-I promoter driving GUS. No expression was found in leaves, however GUS expression was present in the root meristems and root hairs.

Fig 18 shows histochemical staining of a second generation transgenic tobacco seedling, transformed with the S1-L promoter driving GUS. Expression is strong throughout the vascular tissue.

Fig 19 shows histochemical staining of a transgenic banana transformed with the S1-L promoter driving GUS. Like the S1-L promoter in tobacco, GUS expression in the leaves (Fig 19a) and roots (Fig 19b) was limited to the vascular tissue.

Fig 20 shows histochemical staining of a transgenic banana transformed with the S2-I promoter driving GUS. Like the S2-I promoter in tobacco, GUS expression was present in the root meristems. Of ten regenerants (ten different lines), only this line showed GUS expression.

Fig 21 shows histochemical staining of a transgenic banana transformed with S1-I promoter -ubiquitin intron driving GUS. Expression was much stronger than that of the S1-L promoter. Expression appears to still be restricted to the vascular tissue of the leaves (Fig. 21a), however, tissue specificity cannot be identified in the roots due to the high level of GUS expression (Fig. 21b). Results with the S2-I promoter and ubiquitin intron were identical.

Fig 22 shows the DNA sequence of the S1 promoter fragment which is derived from BBTV-S1 and includes 335bp of the open reading frame (ORF) (in bold) and a 259 bp intergenic region. The 594 bp sequence is the S1L promoter. The 287 bp S1I promoter (in italics) includes the intergenic region and 28bp of the ORF 5'. The 116 bp S1P promoter fragment (underlined) is a truncation of the S1I promoter fragment.

Fig 23 shows the DNA sequence of the S2I promoter (268 bp) (italics) and S2-L promoter (900 bp) in bold derived from BBTV S2. The 98 bp S2P promoter fragment (underlined) is a truncation of S2I.

Fig 24 shows a summary of the characteristics of S1 and S2 promoters. The results are based on studies using the GUS reporter gene in stably transformed tobacco and banana. The "Promoter Strength" section is based on histochemical results

in transgenic banana. GUS expression was quantitated using a scale from 0 to 10, in which 10 was the strongest and represents expression of the S1 and S2 promoters with ubiquitin intron. Promoter strength studies in tobacco yielded similar results, except the constructs with the ubiquitin intron showed very low expression (intron no spliced out in dicots). The final column shows the tissue specificty of expression, where Lvt is leaf vascular tissue, Rvt is root vascular tissue, Rm is root meristem, Rh is root hair, Rc is root constitutive expression and Pi is pollen.

EXPERIMENTAL

1. BBTV 6 PROMOTER SEQUENCES (pBT6)

1.1 Materials and Methods

1.1.1 Isolation of BT6 promoter sequences

The nucleotide sequence of BBTV DNA-6 has been published [Burns TM, Harding RM, Dale JL: The genome organisation of banana bunchy top virus: analysis of six ssDNA components. J Gen Virol 76: 1471-1482 (1995)]. For convenience, the A in the ATG translational start codon of the major virion sense ORF has been designated position +1. Molecular techniques were essentially as described by Sambrook et al. [Sambrook J, Fritsch EF, Maniatis T: Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989)]. The largest potential promoter sequence, designated BT6.1, was isolated by PCR amplification from a nucleic acid extract from BBTV infected bananas [Harding RM, Burns TM, Dale JL: Virus-like particles associated with banana bunchy top disease contain small single stranded DNA. J Gen Virol 72: 225-230 (1991)], using two oligonucleotides: primer 1, 5'-CTGCAGAGTTGTGCTGTAATGTT-3' (BBTV DNA-6 sequence from nt -623 to -607 with a PstI restriction site at the 5' end) and primer 2, 5'-GGATCCGCTTCGTCCTTCCGCT-3' (BBTV DNA-6 sequence complementary to nt -1 to -17 with a BamHI restriction site at the 5' end). The PCR reaction contained 20 pmol of each primer, with 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl pH 8.0, 200 µM dNTPs, 0.2 U AmpliTaq polymerase, and 1 µL of a 1/1000 dilution of nucleic acid extract. The reaction mix was subjected to an initial denaturation step of 94° C for 5 min, followed by 30 cycles of 94° C for 1 min, 45° C for 30 sec, 72° C for 1 min, and a final extension step of 72° C for 10 min. The amplified product was cloned into pGEM-T vector and subsequently into PstI/BamHI digested pUC19 as a PstI/BamHI fragment (pUC-BT6.1). The nucleotide sequence was confirmed using an Applied Biosystems 373A DNA sequencer. Promoter BT6.1 was 623 bp and encompassed nt -1 to -623.

The first 5' deletion of BT6.1 was generated using a unique AccI restriction site present at nt -353 to -348. Clone pUC-BT6.1 was digested with AccI, which was present in both the pUC19 multiple cloning site and BT6.1, and subsequently blunted using DNA polymerase I large (Klenow) fragment. The resulting digestion was re-ligated to produce BT6.2, which comprised 351 bp and encompassed nt -1 to -351. Promoter BT6.3 was isolated by PCR amplification using oligonucleotides primer 2 and primer 3, 5'- CTGCAGCATGACGTCAGCAAGG-3' (BBTV DNA-6 sequence from nt -239 to -224 with a PstI restriction site at the 5' end). BT6.3 was amplified, cloned and sequenced as previously described. BT6.3 was 239 bp and encompassed nt -1 to -239. The final 5' deletion was generated using a HaeIII restriction site present at nt -185 to -182. Clone pUC-BT6.1 was digested with HaeIII and BamHI. The resulting sequence was cloned into HincII/BamHI digested pUC19. BT6.4 was 183 bp and encompassed nt -1 to -183. BT6.5, a 3' deletion of BT6.1, was produced by PCR amplification using oligonucleotides: primer 1 and primer 4, 5'-GGATCCTGGACCGGGCTT-3' (BBTV DNA-6 sequence complementary to nt -147 to -162 with a BamHI restriction site at the 5' end). BT6.5 was amplified, cloned and sequenced as previously described. BT6.5 was 476 bp and encompassed nt -147 to -623.

1.1.2 Construction of BT6-uidA fusions

Each of the five BT6 sequences were cloned as HindIII/BamHI fragments upstream of the uidA reporter gene in the binary vector pBI101.3. These constructions were designated pBT6.1 to pBT6.5. Vectors pBT6.1, pBT6.2, and pBT6.3 were subsequently used for Agrobacterium-mediated transformation of tobacco. For micro-particle bombardment studies, the BT6-uidA-nos cassette from each construction was cloned as a HindIII/EcoRI fragment into HindIII/EcoRI digested pGEM-3zf$^+$. These constructions were designated pGEM6.1-GN to pGEM6.5-GN. The 35S-uidA-nos cassette from pBI121 was similarly cloned (pGEM35S-GN), as a positive control for NT-1 transient assays. For promoter comparisons in banana embryogenic cells, the vectors pUGR73, pGUS-2, and pGEM-Adh-GN were used. Plasmid pUGR73 consists of the maize ubi1 promoter

and intron [Christensen AH, Quail PH: Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants. Transgenic Res 5: 213-218 (1996)] located upstream of a uidA reporter gene and 3' rbcS untranslated region in pBluescript. pGUS-2 contains a 530 bp CaMV 35S promoter upstream of a uidA-35S 3' cassette in pUC18. pGEM-Adh-GN was generated by excision of the maize adh1 promoter and intron from pEMU-MCS [Last DI, Brettell RIS, Chamberlain DA, Chaudhury AM, Larkin PJ, Marsh EL, Peacock WJ, Dennis ES: pEMU: an improved promoter for gene expression in cereal cells. Theor Appl Gen 81: 581-588 (1991)] as a SalI/XbaI fragment and insertion upstream of a uidA-nos cassette in pGEM3zf$^+$.

1.1.3 Construction of BT6-NPTII fusion

The plasmids pTAB5 has a 35S 5'-NPTII-35S 3' cassette in pBIN based vector. The NPTII gene and CaMV 35S 3' untranslated region were excised from pTAB5 as a KpnI fragment, and cloned into the KpnI site of pGEM-3zf$^+$ (pGEM-NPT). The BT6.3 promoter (239 bp) was excised from pUC-BT6.3 as a HindIII/BamHI fragment and cloned into the HindIII/BamHI restriction sites located upstream of the NPTII gene in pGEM-NPT to generate the vector pGEM6.3-NPT. The BT6.3-NPTII-35S 3' cassette was excised from pGEM6.3-NPT as a PstI/EcoRI fragment and subcloned into the PstI/EcoRI sites of pBluescript (pBS-BT6.3-NPT). The BT6.3-NPTII-35 3' cassette was subsequently excised from pBS-BT6.3-NPT as a SpeI/EcoRI fragment and cloned into the SpeI/EcoRI sites of pTAB5 to replace the original 35S-NPTII-35S cassette and generate the construct pTAB6.3-NPT. In the final cloning step, the 35S 5'-uidA-35S 3' cassette from pGUS2 was excised as an EcoRI fragment and inserted into a unique EcoRI site in pTAB6.3-NPT. This construct was designated pBT6.3-NPT and was subsequently used for Agrobacterium-mediated transformation of tobacco.

1.1.4 Construction of BT6-GFP fusion

The vector blue-SGFP-TYG-nos (SK) contains a synthetic gene encoding the green fluorescent protein (GFP) cloned

upstream of a nos terminator in pBluescript based plasmid [Chiu WL, Niwa Y, Zeng W, Hirano T, Kobayashi H, Sheen J: Engineered GFP as a vital reporter in plants. Curr Biol 6: 325-330 (1996)]. The GFP-nos cassette from pblue-SGFP-TYG-nos was initially subcloned as a BamHI/KpnI fragment into BamHI/KpnI digested pGEM3zf$^+$. This vector was designated pGEM-GFP. The GFP-nos cassette was subsequently excised from pGEM-GFP as a BamHI/SacI fragment and inserted into the BamHI/SacI restriction sites of pGEM6.1-GN to replace the original uidA reporter gene. This construction was designated pGEM6.1-GFP. A CaMV 35S-GFP fusion was similarly generated, by excision of the GFP-nos cassette from pGEM-GFP as a BamHI/SacI fragment and insertion into the BamHI/SacI restriction sites of pGUS-2, to replace the original uidA-35S 3' cassette. This construction was designated p35S-GFP. A maize ubi-1-GFP fusion was generated by insertion of the maize ubi-1 HindIII/BamHI promoter fragment into the HindIII/BamHI restriction sites located upstream of the GFP gene in pGEM-GFP. This construction was designated pGEM-Ubi-GFP.

1.1.5 Agrobacterium-mediated transformation

Constructs used for Agrobacterium-mediated transformation included pBT6.1, pBT6.2, pBT6.3 (uidA fusions) and pBT6.3-NPT (NPTII fusion). Positive controls for these studies included pBI121 (nos-NPTII-nos/35S-uidA-nos), and pTAB6 (35S-NPTII-35S/35S-uidA-35S). All BT6 promoter fusions and positive controls were introduced into Agrobacterium tumefaciens strain LBA4404 by electroporation [Singh A, Kao TH, Lin JJ: Transformation of Agrobacterium tumefaciens with T-DNA vector using high-voltage electroporation. Focus 15: 84-87 (1993)]. Agrobacterium harbouring the plasmids were used to infect Nicotiana tabacum cv. Xanthii leaf discs, essentially as described by Horsch et al. [Horsch RB, Fry J, Hoffmann N, Neidermeyer J, Rogers SG, Fraley RT: Leaf disc transformation. In Gelvin SB, Schilperoort RA (eds) Plant Molecular Biology Manual, pp A5/1-A5/9. Kluwer Academic Publishers, Dordrecht, Netherlands (1988)]. Transformed tobacco plants were regenerated on media containing kanamycin (100 mg/L) and

timentin (200 mg/L). At least seven independent transgenic lines of tobacco were obtained for each promoter construct. Leaves from plants transformed with the BT6-uidA fusions (1 month old) were transferred to Murashige and Skoog (MS) media [Murashige T, Skoog F: A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 15: 473-497 (1962)] containing 1 mg/L BAP and 0.1 mg/L NAA to induce callus formation.

1.1.5 Preparation of Tissues for Bombardment

Tobacco cells: A Nicotiana tabacum cell line (NT-1) was used [An G: High efficiency transformation of cultured tobacco cells. Plant Physiol 79: 568-570 (1985)]. Ten days prior to bombardment the NT-1 cell line was subcultured at high density in NT media as described by An [An G: High efficiency transformation of cultured tobacco cells. Plant Physiol 79: 568-570 (1985)]. Three days prior to bombardment, the NT-1 cells were harvested by low speed centrifugation. Aliquots of 400 µL of NT cell suspension, containing packed cells / liquid NT medium ratio of 1:1 v/v, were dispersed onto NT media solidified with 0.7% agar (Sigma) and air dried for 2-3 hrs.

Banana cells: Embryogenic suspension cultures of Musa spp., cv. Bluggoe were initiated and maintained as described by Dhed a et al. [Dhed a D, Dumortier F, Panis B, Vuylsteke D, De Langhe E: Plant regeneration in cell suspension cultures of the cooking banana cv. Bluggoe (Musa spp., ABB group). Fruits 46: 125-135 (1991)]. Cells were subcultured fortnightly in maintenance medium [Dhed a D, Dumortier F, Panis B, Vuylsteke D, De Langhe E: Plant regeneration in cell suspension cultures of the cooking banana cv. Bluggoe (Musa spp., ABB group). Fruits 46: 125-135 (1991)] containing 7.5 µM 2, 4-D and 1 µM zeatin. Five days post subculture, cell suspensions were filtered through a 450 µm filter and harvested by low speed centrifugation. Aliquots of 200 µL of a cell suspension, containing packed cells / liquid media ratio of 1:5 v/v, were placed onto 70 mm filter paper discs (Whatman #1) to form a thin layer of evenly dispersed cells. Discs were stored on BH

media solidified with 0.7% agar for 5 days prior to bombardment.

Explants: Whole leaves from Nicotiana tabacum cv. Xanthii and banana cv. Williams (Musa spp. AAA group) plants maintained in tissue culture were excised immediately prior to bombardment. Leaf explants were arranged centrally on 9 cm Petri dishes containing basic MS media solidified with 0.7% agar.

1.1.6 Particle bombardment

All plasmids used for micro-particle bombardment were purified using QIAGEN Maxi Plasmid Purification columns according to manufacturers specifications. Tissue was bombarded using a particle inflow gun [Finer JJ, Vain P, Jones MW, McMullen MD: Development of the particle inflow gun for DNA delivery to plant cells. Plant Cell Reports 11: 323-328 (1992)]. Preparation of 1.0 µm microcarrier gold particles, and coating of plasmid DNA were essentially as described by Mahon et al. [Mahon RE, Bateson MF, Chamberlain DA, Higgins CM, Drew RA, Dale JL: Transformation of an Australian variety of Carica papaya using microprojectile bombardment. Aust J Plant Physiol 23: 679-685 (1996)], except that 2 µg of plasmid DNA was used to coat gold particles and 5 µL of the DNA-gold suspension was used for each bombardment. Target tissues were placed on a platform 7.5 cm from point of particle discharge and covered with a protective baffle of 210 µm stainless steel mesh during bombardment. Tissue was bombarded in a chamber evacuated to 600 mm Hg and particles delivered with a helium pressure of 550 kPa.

For NT-1 transient expression studies, one plasmid-gold suspension was prepared for each promoter fusion. This suspension was used to bombard five NT-1 plates. Three days post bombardment, GUS activity was assayed in one plate by GUS histochemical analysis, and in the remaining 4 plates using the GUS fluorometric assay. This experiment was repeated twice. The results from the GUS fluorometric assays were pooled and statistically analysed using SPSS for Windows V6.1 statistics program.

For banana transient GUS expression studies, one plasmid-gold suspension was prepared for each promoter fusion. This suspension was used to bombard six plates of banana cells. Three days post bombardment, GUS activity was assayed in one plate by histochemical GUS analysis, and in the remaining five plates by fluorometric analysis. This experiment was repeated twice. Results were pooled and analysed as previously described. Transient GFP studies were performed similarly, except GFP expression, presented as number of green fluorescent foci, was visualised with a Leica GFP stereo-microscope. Tobacco and banana leaf explants were bombarded with promoter fusions and GUS activity assayed by histochemical analysis or GFP expression visualised three days post bombardment.

1.1.7 Histochemical GUS localisation

GUS activity was assayed histochemically essentially as described by Jefferson et al. [Jefferson RA, Kavanagh TA, Bevan MW: GUS fusions: -glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6: 3901-3907 (1987)]. Tobacco leaf, stem and root sections from primary transformants were immersed in GUS reaction solution containing 100 mM sodium phosphate buffer (pH 7.1), 50 mM ascorbic acid, 0.1% Triton X-100 and 1 mM 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc). On some occasions, vacuum infiltration prior to incubation was used to enhance penetration of stain into tissues. Bombarded NT-1 cells, banana embryogenic cells on filter paper and tobacco explants were transferred to microtitre trays and immersed in GUS reaction solution. Tissues were incubated at 37^{0}C for 12-16 hrs, and cleared in ethanol:acetic acid solution (5:1).

1.1.8 Fluorometric GUS assay

Bombarded NT-1 and Bluggoe cells were harvested and lysed by sonication for 20 sec in GUS extraction buffer [Jefferson RA, Kavanagh TA, Bevan MW: GUS fusions: -glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6: 3901-3907 (1987)]. Protein concentration of the clarified extract was determined by the method of Bradford

[Bradford MM: A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye-binding. Anal Biochem 72: 248-254 (1976)]. GUS activity was determined using 4-methyl-umbelliferyl- -D-glucuronide (MUG, Sigma) as the substrate, essentially as described by Jefferson [Jefferson RA, Kavanagh TA, Bevan MW: GUS fusions: -glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6: 3901-3907 (1987)]. Fluorescence was measured using a Perkin Elmer LS50B luminescence spectrophotometer. GUS activities were expressed as pmol methylumbelliferone (MU) per min per mg total protein.

1.1.9 NPTII ELISA

Levels of neomycin phosphotransferase II (NPTII) in leaves and roots of primary transformants were quantified using a NPTII ELISA kit (5 Prime → 3 Prime, Inc.). Leaves and roots from two month old tissue culture plantlets were harvested and protein extracted by homogenisation in NPTII extraction buffer containing 0.25 M Tris-HCl pH 7.8, and 1.0 mM phenylmethylsulfonylfluoride (PMSF). Protein concentration of the clarified extract was determined by the method of Bradford [Bradford MM: A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye-binding. Anal Biochem 72: 248-254 (1976)]. The samples were analysed by ELISA according to manufacturers specifications. Absorbance (= 405 nm) was detected using a Dynatech MR5000 plate reader. Amounts of NPTII present were expressed as ng NPTII per mg total protein.

1.1.10 Visualisation of GFP expression

Expression of GFP in banana embryogenic cells and leaf explants post bombardment were visualised using a Leica MZ12 stereo microscope with GFP-Plus filter module. Number of green fluorescent foci, in banana embryogenic cells, were counted within an eyepiece photoframe region, equivalent to 40 mm^2 on the petri dish, when the objective was set to a magnification of 13.

1.1.11 Insertion of maize ubi1 intron

The vector pGEM6.1-GN consists of the 623 bp BBTV DNA-6 promoter (BT6.1) upstream of the uidA reporter gene and nos 3' UTR in pGEM3zf$^+$. This plasmid contains the maize polyubiquitin 1 (ubi1) promoter and intron [Christensen AH, Quail PH: Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants. Transgenic Res 5: 213-218 (1996), Christensen AH, Sharrock RA, Quail PH: Maize polyubiquitin genes: Structure, thermal perturbation of expression and transcript splicing, and promoter activity following transfer to protoplasts by electroporation. Plant Mol Biol 18: 675-689 (1992)] upstream of the uidA reporter gene and tobacco rbcS 3' UTR. For subsequent cloning the ubi1 promoter and intron was subcloned as a PstI fragment into PstI digested pGEM3zf$^+$. This construction was designated pGEM-Ubi. The 1010 bp ubi1 intron with short upstream exon flanking sequence (41 bp) was excised from pGEM-Ubi as a BglII/SmaI fragment and directionally cloned between the BT6.1 promoter and uidA gene into the BamHI/SmaI sites in pGEM6.1-GN. This construction was designated pGEM6.1-Ubi-GN. The ubi1 intron was similarly inserted between the BT2.1-BT5.1 promoters and the uidA reporter gene to generate the constructs pGEM2.1-Ubi-GN to pGEM5.1-Ubi-GN. Two controls were prepared for this study, the first pUbi -GR consisted of the ubi1 promoter without intron and upstream exon flanking sequence, and was generated by digestion of pUGR73 with BglII/NcoI. The subsequent digestion was blunt ended using DNA polymerase I large (Klenow) fragment and religated. An intron control was generated by removing a 944 nt region harbouring the ubi1 promoter. The promoter region was removed by BglII/BamHI digestion of pUGR73. The subsequent digestion was re-ligated to generate the vector pUbi.INT-GR.

1.1.12 Insertion of maize adh1 intron

The maize adh1 promoter (206 bp) and intron (557 bp) were excised from pEMU-MCS as a SalI/XbaI fragment and inserted into the SalI/XbaI sites upstream of a uidA reporter gene and nos 3'

UTR in pGEM-3zf$^+$ (pGEM-GN). This construction was designated pGEM-Adh-GN. An intron control vector pGEM-Adh.INT-GN was generated by excision of a 557 bp BamHI/SmaI fragment containing the adh1 intron from pEMU-MCS, and insertion into the BamHI/SmaI sites present upstream of the uidA reporter gene in pGEM-GN. The adh1 intron was similarly inserted between the BT6.1 promoter and uidA gene in the vector pGEM6.1-GN. This plasmid was designated pGEM6.1-Adh-GN.

1.1.13 Insertion of sugarcane rbcS intron

A 252 bp sequence incorporating the signal peptide and intron of sugarcane ribulose-1, 5-bisphosphate carboxylase/oxygenase (rbcS) small subunit gene [Tang W, Sun SS: Sequence of a sugarcane ribulose-1, 5-bisphosphate carboxylase/oxygenase small subunit gene. Plant Mol Biol 21: 949-951 (1993)] was amplified from sugarcane (Saccharum officinarum sp.) genomic DNA by polymerase chain reaction using oligonucleotides: primer rbc1, 5'-CCAGCCATGGCGCTCACCGTGATGG-3' and primer rbc2, 5'-GGGCCACACCTGCATCGATGTACG-3'. The PCR reaction contained 20 pmol of each primer, 200 µM dNTPs, 10 ng genomic DNA and 0.5 U Expand^{TM} polymerase with manufacturers buffer system 3. The reaction mix was subjected to an initial denaturation step of 94^{0}C for 5 min, followed by 35 cycles of 94^{0}C for 15 sec, 60^{0}C for 15 sec, 72^{0}C for 1 min, and a final extension step of 94^{0}C for 10 min. The amplified product was cloned into the SmaI site of pBluescript SK and nucleotide sequence verified using an Applied Biosystems 373A DNA sequencer. This vector was designated pBS-rbcS.INT. An intron expression control vector was generated by excision of the rbcS intron and leader sequence from pBS-rbcS.INT as a HindIII/SmaI fragment and insertion into the HindIII/SmaI sites located upstream of the uidA gene and nos 3' UTR in pGEM-GN. This construction was designated pGEM-rbcS.INT-GN. The BT6-rbcS intron fusion was generated by initially removing the 623 bp BT6.1 promoter from pGEM6.1-GN as a HindIII/SmaI fragment and insertion into the HindIII/EcoRV sites located upstream of the rbcS intron in pBS-rbcS.INT. This vector was designated pBS6.1-rbcS. The BT6.1 promoter and rbcS intron were

subsequently excised from pBS6.1-rbcS as a HindIII/SmaI fragment and inserted into the HindIII/SmaI sites located upstream of the uidA reporter in pGEM-GN. This construction was designated pGEM6.1-rbcS-GN.

1.1.13 Insertion of rice actin intron

The plasmid pDM803 contains the rice actin promoter and intron [McElroy D, Zhang W, Cao J, Wu R: Isolation of an efficient actin promoter for use in rice transformation. Plant Cell 2: 163-171 (1990)] upstream of the uidA reporter and tobacco rbcS 3' UTR, in pBluescript II. The rice actin intron (313 bp), including 28 bp of the 5' untranslated exon, and the uidA reporter gene were excised by digestion of DM803 with SacI. The subsequent fragment was inserted into a SacI restriction site located upstream of the nos 3' UTR in pGEM3zf+. The subsequent construction was designated pGEM-Act.INT-GN. The BT6.1 promoter was excised from pGEM6.1-GN as a HindIII/BamHI fragment and cloned into the HindIII/BamHI restriction sites located upstream of the rice actin intron in pGEM-Act.INT-GN. The subsequent construction was designated pGEM6.1-Act-GN.

1.1.14 Generation of intron duplications and combinations

The plasmid pGEM6.1-dUbi-GN consists of duplicated ubi1 introns positioned between the BT6.1 promoter and uidA reporter gene. The ubi1 intron and exon flanking sequence was excised from pGEM-Ubi as a BglII/SmaI fragment and inserted into the BamHI/SmaI restriction sites downstream of the original ubi1 intron in pGEM6.1-Ubi-GN. Vector pGEM6.1-Ubi-Adh-GN consists of the ubi1 and adh1 introns, in tandem, positioned between the BT6.1 promoter and uidA reporter gene. The adh1 intron and exon flanking sequence were excised from pEMU-MCS as a BamHI/SmaI fragment and inserted into the BamHI/SmaI restriction sites downstream of the ubi1 intron in the vector pGEM6.1-Ubi-GN. The plasmid pGEM-Adh-Ubi-GN consists of the adh1 and ubi1 introns, in tandem, positioned between the BT6.1 promoter and uidA reporter gene. The ubi1 intron and upstream exon flanking sequence and uidA reporter gene were excised from

pUbi.INT-GR as an XbaI/SacI fragment and inserted into XbaI/SacI digested pGEM6.1-Adh-GN, to replace the original uidA gene.

1.1.15 Construction of GFP expression cassettes

The vector blue-SGFP-TYG-nos (SK) [Chiu WL, Niwa Y, Zeng W, Hirano T, Kobayashi H, Sheen J: Engineered GFP as a vital reporter in plants. Curr Biol 6: 325-330 (1996)] contains a codon altered gene encoding the green fluorescent protein (GFP) cloned upstream of a nos terminator in pBluescript based plasmid. The GFP-nos cassette from pblue-SGFP-TYG-nos was initially subcloned as a BamHI/KpnI fragment into BamHI/KpnI digested pGEM3zf$^+$. This vector was designated pGEM-GFP. The GFP-nos cassette was subsequently excised from pGEM-GFP as a BamHI/SacI fragment and inserted into the BamHI/SacI restriction sites of pGEM-F/L1-GN and pGEM1.1-GN to pGEM6.1-GN to replace the original uidA reporter gene. These constructions were designated pGEM-F/L1-GFP and pGEM1.1-GFP to pGEM6.1-GFP. A CaMV 35S-GFP fusion was similarly generated, by excision of the GFP-nos cassette from pGEM-GFP as a BamHI/SacI fragment and insertion into the BamHI/SacI restriction sites of pGUS-2, to replace the original uidA-35S 3' cassette. This construction was designated p35S-GFP. A maize ubi1-GFP fusion was generated by insertion of the maize ubi1 HindIII/BamHI promoter fragment into the HindIII/BamHI restriction sites located upstream of the GFP gene in pGEM-GFP. This construction was designated pGEM-Ubi-GFP.

The ubi1 intron was introduced between the BT6.1 promoter and GFP reporter by excision of the GFP gene and nos 3' UTR from pGEM6.1-GFP as a BamHI/SacI fragment and insertion into BamHI/SacI digested pGEM6.1-Ubi-GN, to replace the original uidA reporter. This construction was designated pGEM6.1-Ubi-GFP.

1.1.15 Construction of BT6.3-NPT fusions

Construction of the vector pBT6.3-NPT has previously been described. A 35S 5'-GFP-35S 3' cassette was isolated from pGEM35S-GFP by partial digestion with EcoRI. The fragment was

subsequently inserted into a unique EcoRI site located downstream of the BT6.3-NPT-35S 3' cassette in pGEM6.3-NPT. The resulting construction was designated pGEM6.3-NPT/GFP. Both pBT6.3-NPT and pGEM6.3-NPT/GFP were used for stable transformation of banana cv. Bluggoe.

1.1.16 Isolation and cloning of the BT6 terminator

A 203 bp fragment of BBTV DNA-6 incorporating the polyA and conserved termination signals was PCR amplified from BBTD nucleic acid extract using primer BT6.ter: 5'-GGAGCAGAGACATGGAAGTTAG-3', and primer BT-HOMO.COM: 5'-TACa/tTTTGTCATAGc/tGT-3'. The PCR reaction contained 20 pmol of each primer, 200 µM dNTPs, 1/1000 diluted NA extract and 0.5 U Pfu polymerase with manufacturers buffer system. The reaction mix was subjected to an initial denaturation step of 94^0C for 5 min, followed by 35 cycles of 94^0C for 30 sec, 50^0C for 30 sec, 72^0C for 1 min, and a final extension step of 94^0C for 10 min. The amplified product was cloned into the SmaI site of pBluescript SK (pBS-BT6.ter). The BT6 terminator was subsequently excised from pBS-BT6.ter as a SacI/EcoRI fragment and inserted into the SacI/EcoRI sites of pGEM2.1-GN to pGEM6.1-GN to replace the original nos terminator. The uidA reporter gene was subsequently replaced with the codon altered GFP gene [Chiu WL, Niwa Y, Zeng W, Hirano T, Kobayashi H, Sheen J: Engineered GFP as a vital reporter in plants. Curr Biol 6: 325-330 (1996)] by SmaI/SacI digestion. The resulting constructions were designated pGEM2.1-GFP-BT6 to pGEM6.1-GFP-BT6. Each construction was used for transient transformation of Bluggoe cells via micro-particle bombardment.

1.1.17 Preparation and micro-particle bombardment of banana cells

Embryogenic suspension cultures of Musa spp., cv. "Bluggoe" were initiated and maintained essentially as described by Dhed'a et al. [Dhed a D, Dumortier F, Panis B, Vuylsteke D, De Langhe E: Plant regeneration in cell suspension cultures of the cooking banana cv. Bluggoe (Musa spp., ABB group). Fruits 46: 125-135 (1991)]. All plasmid DNA was

purified using QIAGEN Maxi Plasmid Purification columns and cells bombarded using a particle inflow gun [Finer JJ, Vain P, Jones MW, McMullen MD: Development of the particle inflow gun for DNA delivery to plant cells. Plant Cell Reports 11: 323-328 (1992)]. Bombardment conditions, preparation of DNA-gold suspensions, promoter replicates, and GUS expression analysis were essentially the same as described for banana transient assays.

1.1.18 Histochemical and fluorometric analysis of GUS activity

GUS activity was assayed histochemically as described by Jefferson et al. [Jefferson RA, Kavanagh TA, Bevan MW: GUS fusions: -glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6: 3901-3907 (1987)]. Banana embryogenic cells and explants were immersed in GUS reaction solution containing 100 mM sodium phosphate buffer (pH 7.1), 50 mM ascorbic acid, 0.1% Triton X-100 and 1 mM 5-bromo-4-chloro-3-indoyl glucuronide (X-gluc). Vacuum infiltration prior to incubation was used to enhance penetration of stain into explants. Tissues were incubated at 37^0C for 12-16hrs, and cleared in ethanol:acetic acid solution (5:1). For quantitative GUS assays, bombarded banana cells were harvested and lysed by sonication for 20 sec in GUS extraction buffer [Jefferson RA, Kavanagh TA, Bevan MW: GUS fusions: -glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6: 3901-3907 (1987)]. Protein concentration was determined by the method of Bradford [Bradford MM: A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye-binding. Anal Biochem 72: 248-254 (1976)]. GUS activity was determined using 4-methyl-umbelliferyl- -D-glucuronide (MUG) as the substrate, essentially as described by Jefferson et al. [Jefferson RA, Kavanagh TA, Bevan MW: GUS fusions: -glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6: 3901-3907 (1987)]. Fluorescence was measured using a Perkin Elmer LS50B luminescence spectrophotometer. GUS

activities were expressed as pmol methylumbelliferone (MU) per min per mg total protein. GUS activities from each replicate were pooled and statistically analysed using Statgraphics Statistical Graphics System V 4.0.

1.2 Results

1.2.1 Isolation of the BBTV DNA-6 promoter

Sequence analysis of BBTV DNA-6 identified two putative transcription elements within the intergenic region, a consensus nonanucleotide TATA box positioned 60 nt 5' of the translation start codon, and a GC rich region within the CR-M. The GC rich region within the CR-M has shown similarities to the rightward promoter element (rpe-1) of MSV [Fenoll C, Scharwz JJ, Black DM, Schneider M, Howell SH: The intergenic region of maize streak virus contains a GC-rich element that activates rightward transcription and binds maize nuclear factors. Plant Mol Biol 15: 865-877 (1990)] and the sp-1 mammalian transcription motif [Briggs MR, Kadonaga JT, Bell SP, Tjian R: Purification and biochemical characterisation of the promoter-specific transcription factor, Sp1. Science 234: 47-52 (1986); Kadonaga JT, Jones KA, Tjian R: Specific activation of RNA polymerase II transcription by SP1. Trends Biochem Sci 11: 20-23 (1986)]. Further analysis of the BBTV DNA-6 intergenic region identified other putative promoter motifs (Figure 1). Two regions (nt -287 to -278, and -239 to -230) were identified with strong homology to regions of a 20 bp consensus sequence reported by Bouchez et al. [Bouchez D, Tokuhisa JG, Llewellyn DJ, Dennis ES, Ellis JG: The ocs-element is a component of the promoters of several T-DNA and plant viral genes. EMBO J 8: 4197-4204 (1989)], which contains the 16 bp palindromic ocs-element of Agrobacterium. Both these regions harboured a TGACGT element (nt -287 to -283 and -237 to -233) resembling the tandem repeat sequence of the as-1 transcription enhancer element found in the CaMV 35S [Lam E, Benfey PN, Gilmartin PM, Fang RX, Chua NH: Site-specific mutations alter in vitro factor binding and change promoter expression pattern in transgenic plants. Proc Natl Acad Sci USA 86: 7890-7894 (1989)], FMV [Sanger M, Daubert M, Goodman RM: Characteristics of a strong

promoter from figwort mosaic virus: comparison with the analogous 35S promoter from cauliflower mosaic virus and regulated mannopine synthase promoter. Plant Mol Biol 14: 433-443 (1990)], CVMV [Verdaguer B, de Kochko A, Beachy RN, Fauquet C: Isolation and expression in transgenic tobacco and rice plants, of the cassava vein mosaic virus (CVMV) promoter. Plant Mol Biol 31: 1129-1139 (1996)], RTBV [Bhattacharayya-Pakrasi M, Peng J, Elmer JS, Laco G, Shen P, Kaniewska MB, Kononowicz H, Wen F, Hodges TK, Beachy RN: Specificity of a promoter from the rice tungro bacilliform virus for expression in phloem tissues. Plant J 4: 71-79 (1993), Yin Y, Beachy R: The regulatory regions of the rice tungro bacilliform virus promoter and interacting nuclear factor in rice (Oryza sativa L.). Plant J 7: 969-980 (1995)], and CoYMV [Medberry SL, Lockhart BEL, Olszewski NE: The Commelina yellow mottle virus promoter is a strong promoter in vascular and reproductive tissues. Plant Cell 4: 185-192 (1992)] promoters. An ACGTCA sequence identified at nt -235 to -230 had homology to the hexamer motif of plant histone promoters [Nakayama T, Sakamoto A, Minami M, Fujimoto Y, Ito T, Iwabuchi M: Highly conserved hexamer, octamer and nonamer motifs are positive cis-regulatory elements of the wheat histone H3 gene. FEBS Letters 300: 167-170 (1992), Tabata T, Takase H, Takayama S, Mikam K, Nakatsuka A, Kawata T, Nakayama T, Iwabuchi M: A protein that binds to a cis-acting element of wheat histone genes has a leucine zipper motif. Science 245: 965-967 (1989)]. Two other putative motifs were identified within close proximity of the TATA box: a G-box-like motif, CACGTG (nt -135 to -130), which has been shown to interact with proteins belonging to basic leucine zipper (bZIP) group [Devetten NC, Ferl RJ: Transcriptional regulation of environmentally inducible genes in plants by an evolutionary conserved family of G-box binding factors. Intl J Biochem 26: 1055-1068 (1994), Menkins AE, Schindler U, Cashmore AR: The G-box - a ubiquitous regulatory DNA element in plants bound by the GBF family of bZIP proteins. Trends Biochem Sci 20: 506-510 (1995)], and an associated I-box consensus GATAAG (nt -149 to -144) identified in rbcS gene promoters [Donald G, Cashmore A: Mutation of either G box or I box sequences profoundly

affects expression from the Arabidopsis rbcs-1A promoter. EMBO J 9: 1717-1726 (1990)]. The presence and location of such elements within the BBTV DNA-6 intergenic region and recent evidence of a RNA transcript associated with the major virion sense ORF, suggested the intergenic region functioned in the regulation of transcription.

To possibly increase promoter activity we isolated a fragment which included an untranslated leader region. Promoter BT6.1 (nt -1 to -623), was isolated which included nearly all of the intergenic region between the translation stop and start codons. To determine the regulatory effects of the previously identified transcription elements, four deletions were generated from the BT6.1 promoter (Figure 2). The first of these removed a 272 bp region incorporating the CR-M and GC rich (rpe-1-like) motif to generate the BT6.2 promoter. A second 5' deletion of 112 bp removed one of the TGACGT motifs and the stem and loop structure associated with the CR-SL. This fragment was designated promoter BT6.3. The final 5' deletion removed a 56 bp region harbouring the second TGACGT element and ACGTCA motif to generate the promoter fragment BT6.4. A single 3' deletion removing a 147 bp region containing the TATA box, CACGTG motif and rbcS I-box consensus GATAAG generated the BT6.5 promoter. Fusions of these promoters with the uidA reporter gene were used to compare promoter activities.

1.2.2 Transient activity of the BT6 promoter fragments in tobacco

The BT6-uidA fusions (pGEM6.1-GN to pGEM6.5-GN) were tested for transient activity in tobacco NT-1 cells and leaves via micro-particle bombardment. Using the plasmid pGEM-35S-GN as a positive control, GUS activities from three independent experiments, in which each promoter was bombarded four different times into NT-1 cells, are summarised in Figure 3. The BT6.1 promoter consistently provided expression levels similar to that of the 800 bp CaMV 35S promoter indicating that the BT6.1 promoter was potentially a strong promoter in tobacco undifferentiated cells. Interestingly, the BT6.2 promoter

provided levels of GUS expression which were significantly higher than that of the BT6.1 and CaMV 35S promoters (about 1.5- to 3-fold). A further deletion of 112 bp (promoter BT6.3), produced no significant change in GUS activity. GUS activity associated with the BT6.4 promoter was significantly lower than the BT6.3 promoter (about 2-fold), but not significantly different from that observed with the BT6.1 promoter. A 3' deletion of 147 bp incorporating the TATA box resulted in GUS activity equivalent to that detected for background activity.

To determine the potential expression pattern of the BT6 promoters in tobacco plants, micro-particle bombardment of tobacco leaves was initially tested with pGEM6.1-GN and pGEM6.3-GN. GUS histochemical assays of leaf sections bombarded with either fusion indicated expression was limited to vascular tissue and stomata. Despite the tissue specific pattern of expression displayed by these promoters, the number and intensity of blue foci observed was comparable to that observed with the constitutive CaMV 35S promoter (pGEM-35S-GN), even though expression from this latter promoter was not confined to vascular tissue.

1.2.2 Expression of BT6 promoters in tobacco plants

Plasmids pBT6.1, pBT6.2 and pBT6.3 were used to transform tobacco via Agrobacterium-mediated infection of leaf disks, with the plasmid pBI121 as a positive control. Seven to nine independent transformed lines were obtained for each promoter fusion. Integration of the uidA gene in these plants was confirmed by Southern hybridisation using HindIII digested genomic DNA. Hybridisation signals following exposure to X-ray film indicated each line contained between one and six copies of the uidA gene. GUS activity in primary transformants was assayed histochemically. The majority of transformants (17 from 25 plants) had weak GUS activity limited to the phloem and phloem associated cells of the leaves, stems and roots. The remaining eight plants had no visible GUS expression. Phloem-limited expression was observed with all three BT6 promoter constructs and was in agreement with promoter activity observed

following transient bombardment of tobacco leaves. However, the low levels of GUS expression associated with these plants suggested a significant difference in promoter activity between stable and transient transformation studies. Furthermore, there was a significant reduction in GUS expression in these transformants over time, while maintained in tissue culture conditions. Histochemical GUS assays with leaf and root sections showed no visible GUS expression in the majority of plants tested after one year.

Leaves taken from primary transformants harbouring the BT6-uidA fusions, including plants without visible GUS expression, were transferred to callus induction media. Upon de-differentiation of leaf tissue, callus was excised and GUS activity assayed histochemically. GUS expression was evident in all callus tested, although the level of expression varied between independent lines. In some cases, intensity of GUS expression in the induced callus was visibly comparable to that of callus derived from plants transformed with the CaMV 35S-uidA fusion. The increase in GUS activity between leaf and callus was determined to range from 2- to 100-fold by fluorometric GUS assays. The strong expression displayed by the BT6 promoters in regenerated callus was consistent with GUS expression in six independent NT-1 callus lines stably transformed with the BT6-uidA fusions via Agrobacterium-mediated transformation.

The strength and pattern of expression associated with BT6 promoters suggested they could be potentially useful for the expression of selectable marker genes. To test this, tobacco plants were transformed with a BT6-NPTII fusion. Eight plants transformed with BT6.3-NPTII were obtained via Agrobacterium-mediated transformation of tobacco leaf disks. Transformants were selected on media containing 100 mg/L kanamycin. Presence of the BT6.3 promoter and NPTII gene in primary transformants was confirmed by PCR analysis. Levels of NPTII in the leaves and roots of each independent line were determined by ELISA, and compared to levels obtained from similar tissues of tobacco plants transformed with a nos-NPTII fusion (pBI121) and a CaMV 35S-NPTII fusion (pTAB6) (Figure 4). The levels of NPTII in

both the leaves and roots of the eight BT6.3-NPTII transformants were low (about 2- to 10-fold greater than background) and varied little between independent lines. NPTII levels in both the leaves and roots were comparable to those of three plants harbouring the nos-NPTII fusion, but were considerably lower (about 80-fold in leaves and 40-fold in roots) than levels detected in three plants transformed with a CaMV 35S-NPTII fusion.

1.2.3 Activity of the BT6 promoters in banana cells

The BT6-uidA fusion (pGEM6.1-GN) was tested for transient activity in banana embryogenic cells by micro-particle bombardment. For promoter comparisons, the plasmids pUGR73 (maize ubi-1 promoter and intron), pGUS-2 (530 bp CaMV 35S promoter), and pGEM-Adh-GN (maize adh-1 promoter and intron) were included as controls. GUS activities from three independent experiments, in which each promoter construction was bombarded five times into banana cells are summarised in Figure 5a. Throughout these experiments, the BT6.1 promoter gave very low level of GUS activities, only about 3- to 4-fold greater than the untreated negative control, as determined by fluorometric GUS assays. However, no blue foci were observed following histochemical GUS localisation. Of the other promoters tested, the ubi-1 promoter and intron had the greatest activity (about 170-fold greater than the BT6 promoter). The CaMV 35S promoter was about 4-fold less active than the ubi-1 promoter and intron. GUS activity associated with the adh-1 promoter and intron was not significantly different from the BT6 promoters. The findings with maize ubi-1, adh-1 and CaMV 35S promoters are consistent with previous reports with transient expression in banana Bluggoe cells via micro-particle bombardment [Sagi L, Panis M, Remy S, Schoofs H, De Smec K, Swennen R, Cammue BPA: Genetic transformation of banana and plantain (Musa spp.) via particle bombardment. Bio/Technology 13: 481-485 (1995)].

In contrast, expression from promoter fusions with a synthetic green fluorescent protein reporter gene were very different. The BT6.1-GFP provided levels of expression similar

to that of the maize ubi-1 promoter, as estimated by the number of green fluorescent foci observed following transient bombardment (Figure 5b). The CaMV 35S promoter was consistently the strongest of the three promoters tested with about 1.6-fold the number of green fluorescent foci, in comparison to the BT6 and maize ubi-1 promoters. Banana (cv. Williams) leaves bombarded with the BT6.1-GFP fusion demonstrated strong GFP expression in phloem associated cells only.

1.2.4 Transient assays in banana (cv. Bluggoe) embryogenic cells

Insertion of introns, both independently and in tandem combinations, between the BT6.1 promoter and uidA gene produced varying levels of enhanced GUS activity (see Figure 6). The most significant of these was the enhancing effects of the ubi1 intron. In combination with the BT6.1 promoter (pGEM6.1-Ubi-GN), promoter activity was significantly enhanced from native BT6.1 activity, to levels similar to that of the maize ubi1 promoter and intron (pUGR73). Insertion of the adh1 intron (pGEM6.1-Adh.INT-GN) provided no enhancing effects. Interestingly, the ubi1 intron alone (pUbi-INT-GR) provided strong GUS expression, equivalent to the 35S promoter. The maize ubi1 promoter without intron (pUbi -GR) generated levels of GUS activity similar to the native BT6.1 promoter, suggesting the importance of the intron to promoter activity.

The plasmid pGEM6.1-Ubi-Adh-GN provided about a 10-fold increase in GUS activity from the native BT6.1 promoter, however, was significantly lower than pGEM6.1-Ubi-GN. In contrast the plasmid pGEM6.1-Adh-Ubi-GN generated levels of GUS expression similar to that of pGEM6.1-Ubi-GN. Duplication of the ubi1 intron upstream of the BT6.1 promoter provided no significant increase in GUS activity in comparison to the pGEM6.1-Ubi-GN. These results suggest that the enhancing activity of intron combinations is primarily determined by the intron positioned immediately 5' of the transgene.

The sugarcane rbcS intron alone (pGEM-rbcS.INT-GN) provided low levels of GUS expression, about 2-fold greater

than background. In combination with the BT6.1 promoter (pGEM6.1-rbcS-GN), however, the sugarcane rbcS intron enhanced BT6.1 promoter activity approximately 10-fold more than its native level.

The enhancing effects of the rice actin intron have yet to be quantified by GUS fluorometric assays. Initial studies have demonstrated that the rice actin intron (pGEM-Act.INT-GN) alone drives GUS expression in Bluggoe embryogenic cells to a level similar to that of the CaMV 35S promoter and maize ubi1 intron. The enhancing effects of this intron on the BT6.1 promoter (pGEM6.1-Act-GN) appear similar to that of the enhancing effects of the maize ubi1 intron (pGEM6.1-Ubi-GN), suggesting rice actin intron is processed in a similar manner. These activities are based on the number and intensity of blue foci following transient bombardment as determined by GUS histochemical assays.

The BT6.1 and maize ubi1 or the rice actin intron combinations may be potentially useful for the over-expression of transgenes in banana. However, the tissue specificity of these promoter-intron combinations must first be evaluated in transgenic banana plants.

1.2.5 GFP expression in banana cells

Transient assays with BT promoter-GFP fusions in banana (cv. Bluggoe) demonstrated detectable levels of expression from each promoter construction (except the F/L1 promoter). In contrast to GUS transient assays, GFP expression was visibly detectable. This difference in reporter expression most likely reflects a greater sensitivity for detection of the GFP reporter due to altered codon usage and enhanced chromophore formation of the GFP apoprotein [Chiu WL, Niwa Y, Zeng W, Hirano T, Kobayashi H, Sheen J: Engineered GFP as a vital reporter in plants. Curr Biol 6: 325-330 (1996)]. Levels of promoter activity from the BT promoter-GFP fusions were determined by comparing the number of green fluorescent foci observed in a fixed area following transient bombardment of banana embryogenic cells (see Figure 7). The greatest number of green fluorescent foci were observed with the BT4.1 and

BT5.1 promoters. Promoters BT3.1 and BT6.1 were significantly lower (about 66%), promoter BT1.1 (about 30%) and promoter BT2.1 (about 15%). No green fluorescent foci were observed with the BT-F/L1 promoter fusion, possibly suggesting this promoter is trans-activated by a BBTV-encoded protein or the promoter is highly tissue specific.

The BT6 terminator can act as a suitable alternative to the commonly used nos terminator. The plasmids pGEM2.1-GFP-BT6 to pGEM6.1-GFP-BT6 were bombarded into bluggoe cells and assessed for GFP fluorescence two days post bombardment. Levels of GFP expression (ie. number and intensity of green fluorescent foci) were comparable to the plasmids pGEM2.1-GFP to pGEM6.1-GFP, which harbour the nos terminator.

1.2.6 Stable transformation of banana with BT promoter constructs

Transgenic banana cv. Bluggoe plants harbouring the BT promoter -GUS fusions (pGEM1.1-GN to pGEM6.1-GN) and two introns (pGEM6.1-Ubi-GN and pGEM6.1-Adh-GN) have been established. Between 5 and 15 banana plants have been obtained for each promoter construction. To date, plants harbouring the BT6.1-uidA fusion have been screened for presence of the uidA reporter by PCR, and GUS histochemical assays have detected no visible GUS expression, suggesting the BT6.1 promoter is probably weakly active in its host species. Four transgenic banana plants harbouring pGEM6.1-Ubi-GN demonstrate strong GUS expression with the GUS histochemical assay, suggesting the enhancing effects of the maize ubi1 intron are maintained in the transition from transient to stable expression. Greatest expression is evident at the cut surfaces of leaf explants and the throughout the roots. Plants harbouring the plasmid pGEM6.1-Adh-GN have show no evidence of GUS expression after histochemical assays. This may reflect the weak enhancing activity of this intron as demonstrated in transient assays.

Transgenic banana plants harbouring the plasmids pGEM6.1-GFP and pGEM6.1-Ubi-GFP have been regenerated. Between 4 and 10 plants have been obtained for each construction. Fluorescence is maintained in stable regenerants and appears

strongest in plants harbouring the pGEM6.1-Ubi-GFP, however, levels and tissue specificity of GFP expression can not be determined at this stage due to the masking effects of chlorophyll-associated autofluorescence. Each of these constructs (except pGEM-F/L1-GFP) have demonstrated varying levels of fluorescence in stably transformed embryos.

Bluggoe plantlets transformed with the plasmid pBT6.3-NPT have been regenerated. At present, 6 plants selected on media containing either 10 mg/L geneticin (active) or 100 mg/L kanamycin, throughout the transformation and regeneration process, have been obtained. Furthermore, stably transformed bluggoe embryos have been obtained harbouring the plasmid pGEM6.3-NPT/GFP. Similar levels of antibiotic were used to select transformants and embryos display varying levels of fluoresce under blue light, suggesting integration of the plasmid. These results suggest the BT6.3 promoter is capable of driving NPTII expression to a level high enough for selection of transformants on either 10 mg/L geneticin (active) or 100 mg/L kanamycin for transformation of banana (cv. Bluggoe).

2. BBTV S1 / S2 PROMOTER SEQUENCE

2.1 Materials and Methods

2.1.1 Plant material

2.1.1.1 Tobacco

A non-regenerable Nicotiana tabacum (NT) cell suspension was maintained as described in An (1985) Plant Physiology 79:568-570. For transient assays, cells were collected four days after subculturing and cells allowed to settle. Sufficient supernatant was removed to leave a packed cell volume/liquid medium ratio of approximately 1:1. Cells were then resuspended and dispensed in 200 mL aliquots onto 70 mm diameter Whatman filter paper discs in 90 mm petri dishes containing NT media with 7 g/L Sigma washed agar. Cells were bombarded immediately following plating.

Tobacco (Nicotiana tabaccum var xanthi) plantlets were maintained for Agrobacterium-mediated transformation. The

tobacco were grown on MS media (Murashige and Skoog (1962) Plant Physiology 15:473-497) and subcultured monthly.

2.1.1.2 Banana

Embryogenic cell suspensions of banana (cv. 'Bluggoe' Musa spp. ABB) were initiated and maintained essentially as described by Dhed'a et al (1991) Fruits 46:125-135. Initiation and maintenance media by Dhed'a et al (1991) supra is designated BH medium. Suspension cells were collected four days after subculturing and passed through a 450 mm mesh. The filtrate was then centrifuged at 130 x g for 10 minutes. Sufficient supernatant was removed to leave a packed cell volume/liquid medium ratio of approximately 1:5. Cells were then resuspended and dispensed in 200 mL aliquots onto 70 mm diameter Whatman filter paper discs in 90 mm petri dishes containing BH medium solidified with 7 g/L Sigma washed agar. Cells were bombarded four days after plating.

2.1.2 Promoter constructs

Promoter S1-L (592 bp) was isolated from E502.9 BBTV-S1 clone (Karan (1995) supra) by PCR amplification using primers S1D: 5'-GCAAGCTTGGTCTATGGTCCG-3' (BBTV S1 sequence complementary to nt 885-902 with HindIII restriction site) and S1A 5'-GCGGATCCTGATGACGTAGGG-3' (BBTV S1 sequence from nt 48-62 with BamHI restriction site). The PCR reaction contained 20 pmol of each primer, with 50 mM KCl, 15 mM MgCl2, 10 mM Tris-HCl pH 8.0, 200 mM dNTPs, 0.2 U Amplitaq polymerase, and ~ 0.1 ng of BBTV nucleid acid. The reaction mix was subjected to an initial denaturation step or 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 45°C for 30 s, 72°C for 1 min, and final extension step of 72°C for 10 min. The amplified product was cloned into pGEM-T and subcloned as a HindIII/BamHI fragment into pBI101.3. This construct was used for Agrobacterium-mediated transformation of tobacco. For microprojectile bombardment, The S1-L promoter was subcloned as a HindIII/BamHI fragment into the pGEM-GN vector, which contained the HindIII/EcoRI fragment from pBI101.3 in HindIII/EcoRI digested pGEM3zf+. The S1-I promoter (285 bp)

was isolated by PCR amplification using primers S1B 5'-GCAAGCTTCCGAAGATAGAATAAAG-3'(BBTV S1 sequence complementary to nt 588-590 with HindIII restriction site) and S1A. The S1-P promoter (114 bp) was isolated by PCR amplification using primers S1C 5'-GCAAGCTTAGCCACGAAGGAAC-3' (BBTV S1 sequence complementary to nt 1056-1073 with HindIII restriction site) and S1A.

Promoter S2-I (268 bp) was isolated from E424.T2 BBTV-S2 clone (Karan 1995 supra) by PCR amplification using primers S2A: 5'-GCGTCGACAGAAGATAGAATA-3' (BBTV S2 sequence complementary to nt 890-903 with SalI restriction site) and primer S2C 5'-GCTCTAGATGATGACGTCAGGG-3' (BBTV S2 sequence from nt 48-63 with XbaI restriction site). The PCR reaction was identical to that of the S1-L promoter. The amplified product was cloned into pGEM-T and subcloned as a SalI/XbaI fragment into pBI101.3. This construct was used for Agrobacterium-mediated transformation of tobacco. For microprojectile bombardment, The S2-I promoter was subcloned as a HindIII/BamHI fragment into the pGEM-GN vector. The S2-P promoter (97 bp) was isolated by PCR amplification using primers S2B: 5'-GCGTCGACGGCCCTTAATGGGCC-3' (BBTV S2 sequence complementary to nt 1061-1076 with SalI restriction site) and primer S2C. The PCR reaction was identical to that of the S1-L promoter. The promoter fragment was treated identically to the S2-I promoter. Promoter S2-L (885 bp) was isolated by PCR amplification using the primers S2D 5'-GCGTCGACGGCTCACTGGGAGA-3' (BBTV S2 sequence complementary to nt 273-286 with SalI restriction site) and primer S2C. The promoter was cloned as a SalI/XbaI fragment into pGEM-GN.

GUS was removed from the S1-I-GN construct with a BamHI/EcoRI digest and replaced with BamHI/EcoRI digested fragment encoding the green fluorescent protein (gfp) from pGEM-GFP. pGEM-GFP was constructed from the vector pblue-SGFP-TYG-nos (Chiu et al (1996) Current Biology 6:325-330). The gfp-nos cassette was subcloned as a BamHI/KpnI fragment into BamHI/KpnI digested pGEM3zf+. S1-L, S1-P, S2-I and S2-P HindIII/BamHI fragments, were cloned into S1-I-GFP digested with HindIII/BamHI.

The 1010 bp Ubi-1 first intron (Christensen and Quail (1996) Transgenic Research 5:213-218) with the short upstream exon flanking sequence (41 bp) was excised from pUGR73 (Ubi-1 promoter/intron-GUS-nos) by a BglII/SacI cut and cloned into S1/2I-GN and S1/2I-GFP digested BglII/SacI vectors.

To compare promoter expression four control plasmids were used, including p35S-GUS, p35S-GFP, pUbi-GUS and pUbi-GFP. p35S-GUS was constructed by removal of the CaMV 35S-GUS-nos cassette from pBI121 by HindIII/EcoRI digestion. This fragment was subcloned into HindIII/EcoRI digested pGEM-3zf+. p35S-GFP was constructed by removing the GUS BamHI/EcoRI fragment from p35S-GUS, and replacing it with a GFP BamHI/EcoRI fragment from pGEM-GFP. pUbi-GFP was constructed using the Ubi-1 promoter and intron fragment was excised from pGEM-Ubi as a HindIII/BamHI fragment and inserted into the HindIII/BamHI sites located upstream of the gfp gene in pGEM-GFP. pGEM-Ubi was constructed by subcloning of the Ubi-1 promoter and first intron from pUGR73 as a PstI fragment into PstI digested pGEM3zf+. For co-transformation experiments equimolar concentrations of the promoter constructs and pDHKan were used (35S-NptII-nos). Plasmids S1/2-I/L/P-GN, S1-I/L/P-GFP and S2-I/P-GFP were used together with pDHKan in co-transformation experiments. Plasmid DNA was prepared using a Bresapure Plasmid Maxi Kit.

2.1.3 Agrobacterium-mediated Transformation

The purified binary vector pBI101.3 containing the potential promoter sequences cloned 5' of the GUS gene (except S2-L) was used to transform Agrobacterium tumefaciens LBA4044 electro-competent cells using a Bio-rad Gene Pulser. Electrocompetent cells were prepared essentially as described by Dower et al (1988) Nucleic Acids Research 16: but cells were grown at 28°C. Transformants were selected at 28°C on LB plates with kanamycin (100 mg/L). Cells containing the plasmid were inoculated into LB with kanamycin (100 mg/L) media and grown overnight at 28°C. This culture was used to transform tobacco leaf discs as described by Horsch et al (1989) Plant Molecular Biology Reporter 5:387-405.

2.1.4 Bombardment conditions

Suspension cells were bombarded using a particle inflow gun (Finer et al (1992) Plant Cell Reports 11:323-328. Gold particles 10 mm in diameter were used as microprojectiles; 120 mg of gold was washed three times in ethanol and three times in sterile distilled water before suspension in 1 mL of sterile 50% (v/v) glycerol. To prepare microprojectiles for bombardment, 25 mL of gold was sonicated for 30 s then mixed with 2 mg of plasmid DNA (equimolar concentration of plasmids when co-transforming), 25 mL 2.5 M $CaCl_2$ and 5 mL 0.1 M spermidine-free base. All solutions were kept on ice. The gold was kept in suspension for 5 min by occasional vortexing, allowed to precipitate for 10 min on ice, and 22 mL of the supernatant removed and discarded. The remaining suspension was vortexed immediately prior to using 5 mL aliquots of the mixture for each bombardment. Target tissue was 75 cm from the point of particle discharge and protected by a 210 mm stainless steel mesh baffle. Helium pressure was 550 Kpa and chamber vacuum was -25 inches Hg. For transient expression studies tissues were assayed 48 hours post bombardment.

2.1.5 Selection and regeneration of plants

2.1.5.1 Tobacco

Kanaymcin (100 mg/L) was used as the selective agent, and was present in all media after commencement of selection. Timentin (200 mg/L) was also added to remove any residual Agrobacterium. Plantlets were regenerated as described by Horsch et al (1989) supra.

2.1.5.2 Banana

Kanamycin (100 mg/L) was used as the selective agent, and was present in all media after commencement of selection. Cells were subcultured by transferring the supporting filter paper to fresh medium. Bombarded cells were moved to selection media 10 days post bombardment. After three months (monthly subcultures), plants were regenerated as described by Dhed'a et al (1991) supra with one month of regeneration step 1 medium

and one month of regeneration step 2 medium. When transgenic somatic embryos were moved to germination medium (regeneration step 3) they were removed from the filter paper and placed directly on the medium. Transgenic somatic embryos that had formed independently on different parts of the filter paper were kept separate and regarded as independent transformation events. After the formation of roots on regeneration step 4 medium, plants were assessed for reporter gene expression.

2.1.6 GUS assay

For histochemical GUS assays, tissue was incubated in buffer containing 100 mM Sodium phosphate (pH 7.0), 50 mM ascorbate and 1 mM 5-bromo- 4-chloro-3-indolyl-B-D-glucuronide (x-gluc) according to Jefferson (1987). Samples were incubated overnight and cleared with acetic acid:ethanol (1:3). For fluorometric GUS assays, protein was extracted from leaf tissue in GUS extraction buffer (Jefferson (1987) Plant Molecular Biology Reporter 5:387-405) and protein concentration determined by the Bio-rad Protein Assay. GUS activity was assayed according to Jefferson (1987) supra. Values represent the GUS activity in transgenic plants minus the background of non-transformed controls.

2.1.7 GFP assay

GFP expression was visualised using a Leica MZ12 stereo microscope fitted with a Leica GFP Plus fluorescence module. Plant material containing chlorophyll emitted a strong red fluorescence. A green barrier filter (BGG22 Chroma Technology) enabled the visualisation of GFP expression in green plant tissue.

2.2 Results

Potential promoter intergenic regions of these two components, namely S1-L, S1-I, S1-P, S2-L, S2-I and S2-P (Figs 15,22,23) were introduced into constructs to drive the GUS or GFP reporter genes.

The activities of these constructs were assayed in both tobacco and banana and for transient (expression of non-

integrated promoter in cell culture) and stable expression (expression of integrated promoter in regenerated plants).

2.2.1 Transient Activity of Promoter Fragments from BBTV-S1 and S2 in tobacco and banana.

The six promoter sequences, S1-L, S2-L, S1-I, S2-I, S1-P and S2-P driving GUS, were shot into non-regenerable tobacco callus (NT). For each promoter sequence, at least six replicates were shot and the process repeated at least three times. The level of GUS expression was measured 48 h after shooting by both fluorimetric and histochemical means. For histochemical staining, a buffer solution containing X-gluc was layered over the tissue and incubated for 12 hr at 37°C, whilst fluorimetric assays required extraction of protein from the tissue, by sonication, and GUS assays using MUG.

The ratio of promoter activities (as determined by fluorimetric assays) differed somewhat between replicates. However, despite this variation a number of conclusions can be drawn from transient studies. All six promoters showed activity which was equivalent, or slightly less than that of p35SGUS. Of the six promoters, S2-L and S2-I showed the greatest activity, with the other four promoters of approximately equal strength (Figure 14).

In banana tissue, very little activity was detected by histochemical assays, with between 0 and 3 GUS expressing foci per shot. In comparison pUGR73 expressed at high levels in this tissue (200-300 fold). Interestingly, addition of the ubiquitin intron 5' to the GUS gene in S1/2-I-GUS-nos resulted in an increase in expression of around 10-fold, and was equivalent to pUGR73.

2.2.2 Tissue specificity of the promoters in tobacco

The promoters derived from BBTV-S1 and S2 were tested for tissue-specific expression by stably transforming tobacco with the various promoters driving GUS and/subsequent histochemical assay. All regenerated plants were tested for integration by PCR.

In stably transformed tobacco, S1-I and S1-P promoters both expressed weakly in root tissue. No activity was detected histochemically in the leaves. The larger S1-L promoter gave enhanced expression in both roots and vascular tissue of the leaves (Figure 16). GUS expression was present not only in the vascular tissue of the root, but also in root hairs and meristems. GUS expression in roots was stronger than that found in S1-I and S1-P. The increase in expression may indicate the presence of one or more cis-acting elements within the ORF of S1.

The S2-I promoter, like S1-L, expressed strongly in root meristems and hairs (Figure 17). Expression in vascular tissue of the leaves and roots, was weak. Expression was also detected in pollen. No expression was detected in the shot apical meristem.

Seed from these tobacco were germinated. The strongest GUS expression was found with the S1-L promoter, which was active throughout the vascular tissue of the plant (Figure 18).

2.2.3 Tissue specificity of the promoters in banana

The pattern of GUS expression in stably transformed transgenic bananas were similar to that in tobacco. The S1-L promoter showed the strongest expression, and was active in both leaves and roots (Figure 19). Expression appeared to be vascular limited in both the leaves and roots. No visible expression was detected with the S1-I and S1-P promoters. The S2-I promoter also showed little expression, however, one line did express strongly in root meristems (similar to the result with tobacco) (Figure 20). S2-P showed no visible expression. The activity of the S1/2-I promoters increased with the inclusion of the maize ubiquitin intron (Figure 21). Expression was enhanced in both roots and leaves. It was difficult to determine whether the promoter remained vascular limited.

Replacing the GUS reporter gene with GFP yielded similar results. However, expression from the weaker S1-I, S1-P, S2-I and S2-P could also be visualised. All the promoters appeared to express similarly, with high expression in the roots,

including root meristems and hairs. Expression was also present in the leaves, with greatest amount of GFP expression present in the stomata and vascular tissue.

Transient expression in tobacco callus is reported in Fig. 14. In tobacco callus, the GUS activities from constructs incorporating S1 and S2 promoters were comparable to that from GUS constructs driven by the CaMV 35S promoter (Fig 14). As shown in table 1, the promoters showed different patterns of expression with stable expression in transgenic tobacco plants compared to transient expression. The S1I and S1P promoter fragments expressed weakly in root vascular tissue. The larger promoter fragment (S1L) including 335 bp of the S1 open reading frame (ORF) significantly enhanced expression in both roots and vascular tissue of the leaves and flower (Fig 16) whereas corresponding expression in these tissues was not observed with S1I and S1P promoter fragments. This suggests the presence of one or more cis-acting elements within the ORF of S1L. The S2I promoter fragment expressed strongly in root meristem, root hair and pollen. Expression in vascular tissue of the leaves and roots was weak.

These transgenic tobacco were allowed to flower and the second generation plants germinated from seed. Tobacco with the S1L promoter fragment driving GUS showed strong vascular expression throughout the second generation plant (Figure 18).

With respect to bananas, transient expression of both the S1L and S2I promoter fragments driving GUS was very low, and sometimes undetectable in a banana embryogenic cell suspension. However, the S1I promoter fragment with the inclusion of the ubiquitin intron 5' to the GUS gene showed significant increased expression.

The pattern of GUS expression in stably transformed transgenic bananas (Fig 24) were similar to that in tobacco. This S1L promoter showed the strongest expression, and was active in both leaves and roots (Figure 19). No visible expression was detected with the S1I and S1P promoter fragments. The S2I promoter fragment also showed little expression, but one regenerated plant line did express strongly in root meristems (similar to the result with tobacco) (Figure

20). With the inclusion of the ubiquitin intron, GUS expression increased significantly, with both the S1I and S2I promoter fragments (Figure 21). Replacing the GUS reporter gene with green fluorescent protein (GFP) reporter gene yielded similar results. However, expression from the weaker S1I and S2I promoters could also be visualised.

It will of course be realised that while the foregoing has been given by way of example, all such and other modifications and variations thereto as would be apparent to persons skilled in the art are deemed to fall within the broad scope and ambit of this invention as herein set forth.

CLAIMS

1. An isolated DNA molecule including a promoter sequence derived from a substantially untranslated portion of any one BBTV component and adaptable for promoting transcription of a cloned gene in a plant cell.

2. An isolated DNA molecule as claimed in claim 1 wherein the promoter sequence is derived from BBTV components 6, S1, or S2.

3. An isolated DNA molecule as claimed in claim 1 or 2 wherein the promoter sequence has a DNA sequence selected from the group consisting of:
(i) sequences indicated by pBT6.1-5, S1-L, S1-I, S1-P, S2-I, S2-P;
(ii) sequences complementary to (i); and
(iii) sequences having up to 20% variation from (i) or (ii).

4. An isolated DNA molecule as claimed in any one of claims 1, 2, or 3 wherein the DNA molecule includes a rbcS 1-box consenus region, G-box motif, TATA box, and ATG initiation codon.

5. An isolated DNA molecule as claimed in any one of the above claims wherein the DNA molecule includes a cloned gene for expression in a plant cell.

6. An isolated DNA molecule as claimed in claim 5 wherein the DNA molecule includes an intron located translationally upstream of the cloned gene.

7. An isolated DNA molecule as claimed in claim 6 wherein the intron is the first intron of the 5' untranslated region.

8. An isolated DNA molecule as claimed in claim 6 or 7 wherein the intron is the maize alcohol dehydrogenase 1 (adh1), polyubiquitin 1 (ubi1) or rice actin (act1).

9. An isolated DNA molecule as claimed in any one of claims 5 to 8 wherein the cloned gene is GUS, NPTII, gene conferring insecticide resistance, gene conferring herbicide resistance, or a growth promoting gene.

10. An isolated DNA molecule as claimed in claim 9 and indicated in Figures 2, 6, 9, 10, 11 and 25.

11. An isolated DNA molecule as claimed in any one of claims 6 to 9 wherein there are two or more introns substantially in series.

12. A DNA chimaeric vector or cassette having a DNA molecule as claimed in any one of the claims 5 to 11 wherein the vector or cassette is transformable into a plant cell.

13. A method of expressing a gene in a plant cell using a DNA molecule as claimed in any one of the claims 5 to 11 including

transforming the DNA molecule in a plant cell;

culturing the plant cell with the transformed DNA molecule; and

expressing the cloned gene in the plant cell with the transformed DNA molecule.

14. A method of expressing a gene in a plant cell as claimed in claim 13 wherein the plant cell is a cell from banana, tobacco, cucumber, sugar cane, maize, rice, wheat or corn.

15. A method of expressing a gene as claimed in claim 13 or 14 wherein the plant cell is a cell from a monocotyledon or dicotyledon plant.

16. A plant cell having a DNA molecule as claimed in any one of claims 5 to 11.

17. A plant cell as claimed in claim 16 wherein the plant cell is part of a monocotyledon or dicotyledon plant.

18. A plant having a plant cell as claimed in claims 16 or 17.

19. A plant as claimed in claim 18 wherein the plant is a monocotyledon plant.

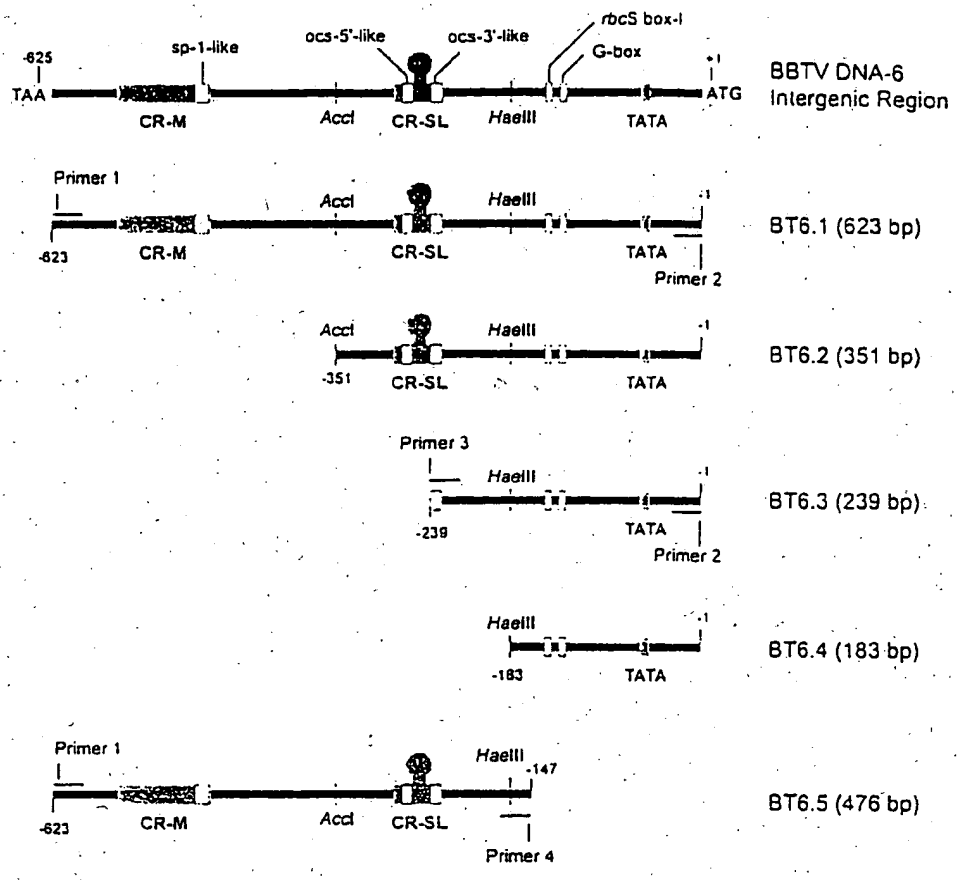
20. A plant as claimed in claim 19 wherein the plant is banana, maize, rice, wheat or corn.

21. A plant as claimed in claim 18 wherein the plant is a dicotyledon plant.

22. A plant as claimed in claim 21 wherein the plant is tobacco.


BT6.1
BT6.5
Primer 1
TAAAAGTTGTGCTGTAATGTTAATTAATAAAACGTATATTTGGG
-600
AAATTGATAGTTGTATAAAACATACAACACACTATGAAATACAA
-550
CR-M
GACGCTATGACAAATGTACGGGTATCTGAATGAGTTTTAGTATC
-500
MSV rpe-1-like motif
GCTTAAGGGCCGCAGGCCCGTTAAAAATAATAATCGAATTATAA
ACGTTAGATAATAATCAGAGATAGGTGATCAGATAATATAAACA
-450
TAAACGAAGTATATGCCGGTACAATAATAAAATAAGTAATAACA
-400
BT6.2
AccI
AAAAAAATATGTATACTAATCTCTGATTGGTTCAGGAGAAAGGC
-350
ocs-like 5' region
CCACCAACTAAAAGGTGGGGAGAATGTCCCGATGACGTAAGCAC
-300
BT6.3
CR-SL
GGGGGACTATTATTACCCCCCGTGCTCGGGACGGGACATGACGT
ocs-like 3' region
-250
Primer 3
CAGCAAGGATTATAATGGGCTTTTTATTAGCCCATTTATTGAAT
BT6.5
-200
Primer 4
HaeIII
TGGGCCGGGTTTTGTCATTTTACAAAAGCCCGGTCCAGGATAAG
BT6.6
-150
rbcS I-box consensus
G-box motif
TATAATGTCACGTGCCGAATTAAAAGGTTGCTTCGCCACGAAGA
-100
AACCTAATTTGAGGTTGCGTATTCAATACGCTACCGAATATCTA
TATA box
TTAATATGTGAGTCTCTGCCGAAAAAAAATCAGAGCGAAAGCGGA
-50
Primer 2
AGGCAGAAGCGATG
+1
BT6.1
BT6.2
BT6.3
BT6.4
Primer
Promoter 5' end
Promoter 3' end

FIGURE 1


rbcS box-I
sp-1-like
ocs-5'-like
ocs-3'-like
G-box
-625
TAA
+1
ATG
CR-M
AccI
CR-SL
HaeIII
TATA
BBTV DNA-6
Intergenic Region
Primer 1
-623
Primer 2
BT6.1 (623 bp)
-351
BT6.2 (351 bp)
Primer 3
-239
BT6.3 (239 bp)
-183
BT6.4 (183 bp)
-147
Primer 4
BT6.5 (476 bp)

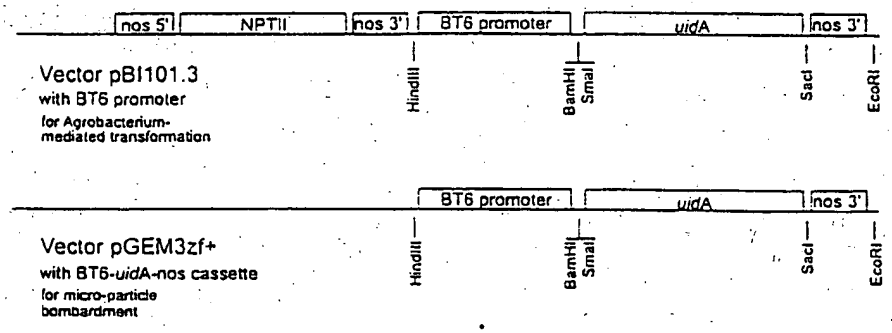

nos 5'
NPTII
nos 3'
BT6 promoter
uidA
Vector pBI101.3
with BT6 promoter
for Agrobacterium-
mediated transformation
HindIII
BamHI
SmaI
SacI
EcoRI
Vector pGEM3zf+
with BT6-uidA-nos cassette
for micro-particle
bombardment

FIGURE 2

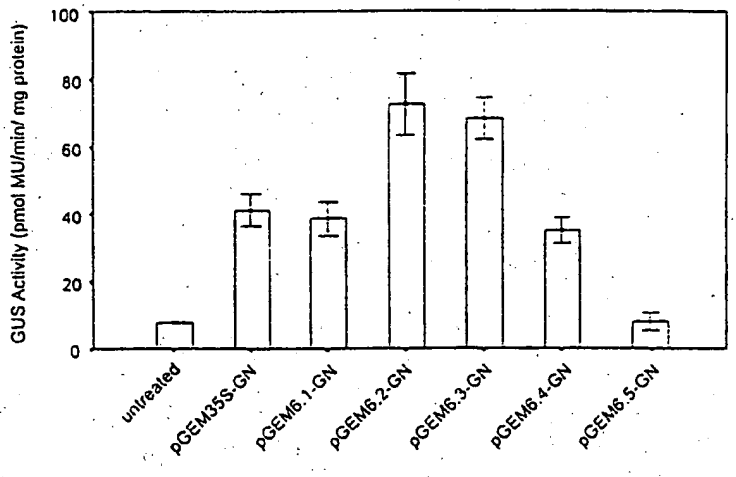

GUS Activity (pmol MU/min/ mg protein)
100
80
60
40
20
0
untreated
pGEM35S-GN
pGEM6.1-GN
pGEM6.2-GN
pGEM6.3-GN
pGEM6.4-GN
pGEM6.5-GN

FIGURE 3

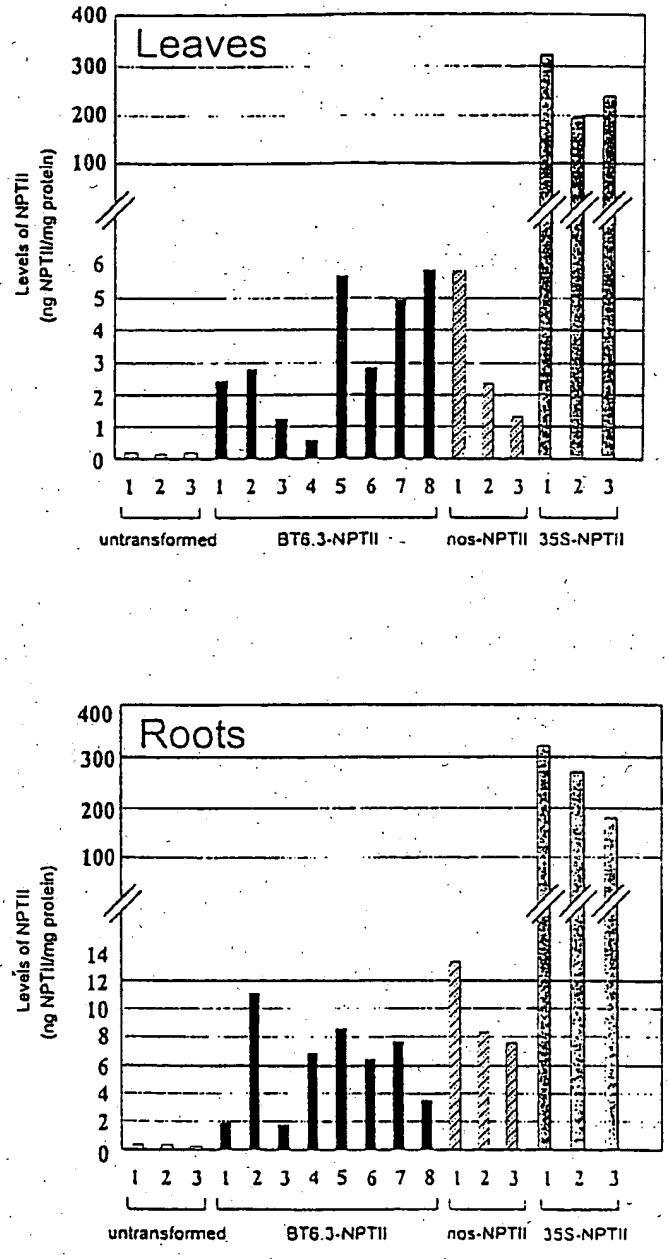

Leaves
Levels of NPTII (ng NPTII/mg protein)
400
300
200
100
6
5
4
3
2
1
0
1 2 3 1 2 3 4 5 6 7 8 1 2 3 1 2 3
untransformed
BT6.3-NPTII
nos-NPTII
35S-NPTII
Roots
Levels of NPTII (ng NPTII/mg protein)
400
300
200
100
14
12
10
8
6
4
2
0
1 2 3 1 2 3 4 5 6 7 8 1 2 3 1 2 3
untransformed
BT6.3-NPTII
nos-NPTII
35S-NPTII

FIGURE 4

(a)

Promoter	Transient GUS activity (pmol MU/min/mg total protein)
untreated	13.1 ± 0.9 [a]
BT6.1	47.5 ± 5.2 [a]
maize *adh*1	38.6 ± 3.3 [a]
CaMV 35S	2231.2 ± 174.6 [b]
maize *ubi*1	8560.5 ± 194.7 [bc]

(b)

Promoter	Transient GFP Expression (number green fluorescent foci)
BT6.1	75.2 ± 4.1 [a]
maize *ubi*1	73.9 ± 4.0 [a]
CaMV 35S	119.7 ± 2.9 [b]

FIGURE 5

Promoter Construction	GUS Activity (pmol MU/min/mg protein)
untreated	13.1± 0.9
pGEM6.1-GN	47.5 ± 5.2
pGUS2	2231.2 ± 174.6
pUGR73	8560.5 ± 194.7
pUbi -GR	60.8 ± 10.6
pUbi.INT-GR	1071.0 ± 75.6
pGEM-Adh-GN	38.6 ± 3.3
pGEM-Adh.INT-GN	25.0 ± 1.1
pGEM6.1-Ubi-GN	6416.5 ± 324.6
pGEM6.1-Adh-GN	156.2 ± 26.0
pGEM6.1-Ubi-Adh-GN	547.9 ± 45.2
pGEM6.1-Adh-Ubi-GN	6259.8 ± 402.4
pGEM-dUbi-GN	8405.7 ± 728.1
pGEM-rbcS.INT-GN	29.2 ± 1.4
pGEM6.1-rbcS-GN	526.8 ± 39.6

FIGURE 6

Promoter Construction	Number of green fluorescent foci
pGEM-F/L1-GFP	none
pGEM1.1-GFP	24.4 ± 1.3
pGEM2.1-GFP	15.3 ± 1.2
pGEM3.1-GFP	59.3 ± 5.2
pGEM4.1-GFP	88.6 ± 3.9
pGEM5.1-GFP	92.0 ± 4.7
pGEM6.1-GFP	55.0 ± 1.5

FIGURE 7

GGAGCAGAGACATGGAAGTTAGTATTAGTAACAGCAACAACTGTAATGAATTATGTGATCTGA
AGTGTTATGTTGTTTGTTCGTTAAGAATCAAGGAATAAAAGTTGTGCTGTAATGTTAATTAAT
AAAACGTATATTTGGGAAATTGATAGTTGTATAAAACATACAACACACTATGAAATACAAGAC
GCTATGACAAATGTA

FIGURE 8

FIGURE 9

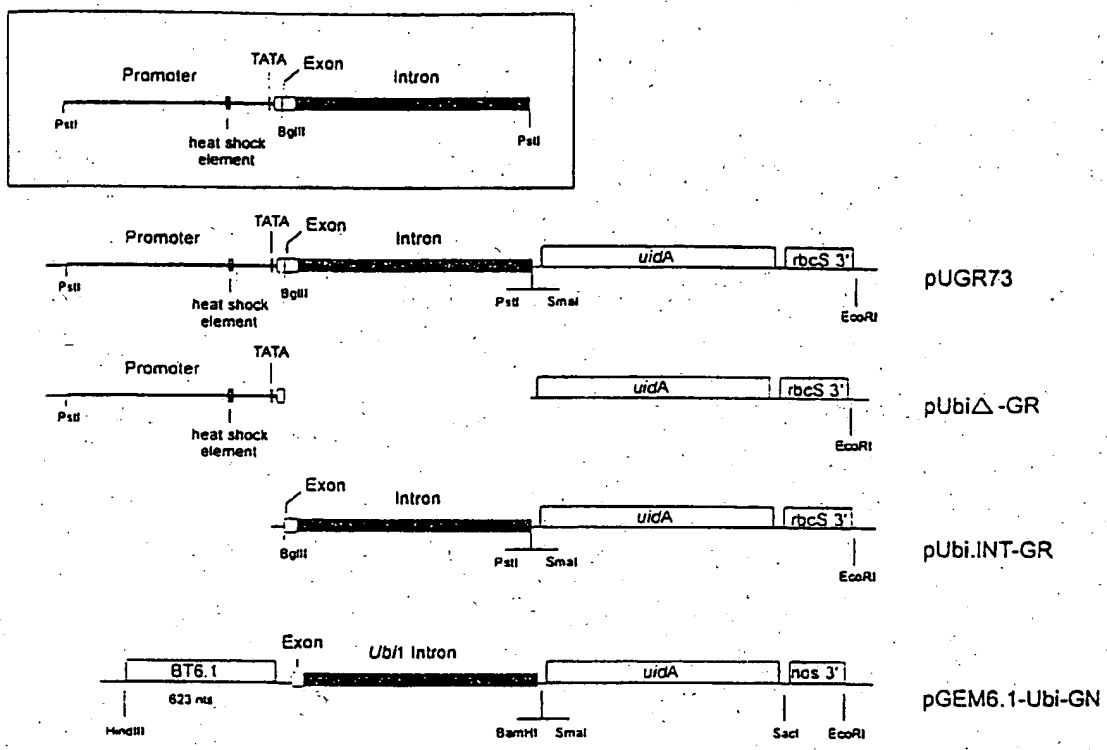


FIGURE 10

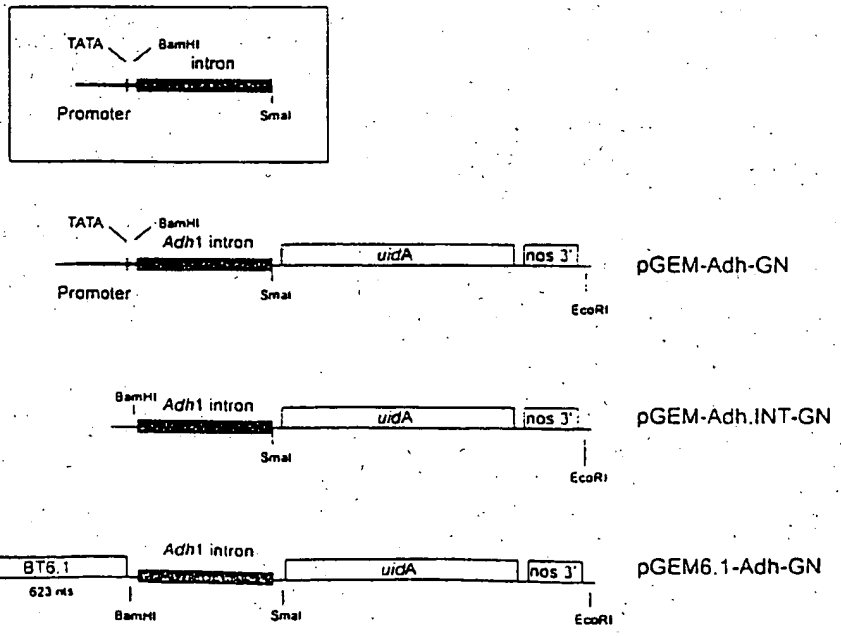
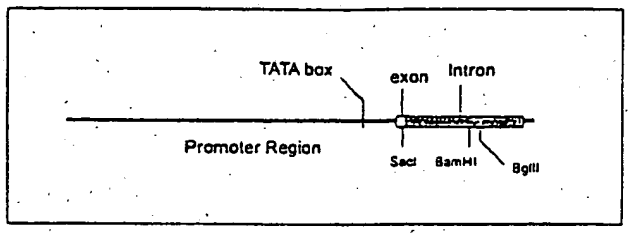
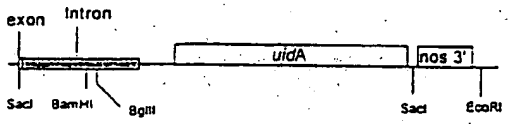


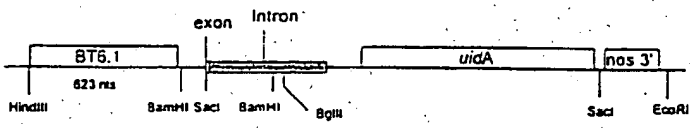
FIGURE 11



pGEM-Act.INT-GN



pGEM6.1-Act-GN



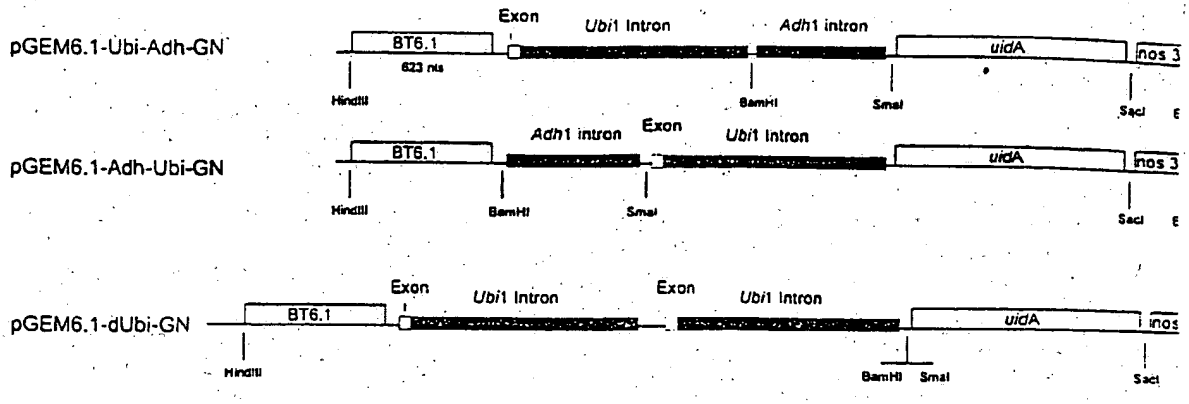

pGEM6.1-Ubi-Adh-GN
Exon
Ubi1 Intron
Adh1 intron
BT6.1
623 nts
uidA
nos 3
HindIII
BamHI
SmaI
SacI
pGEM6.1-Adh-Ubi-GN
Adh1 intron
Exon
Ubi1 Intron
pGEM6.1-dUbi-GN
Exon
Ubi1 Intron
Exon
Ubi1 Intron

FIGURE 12

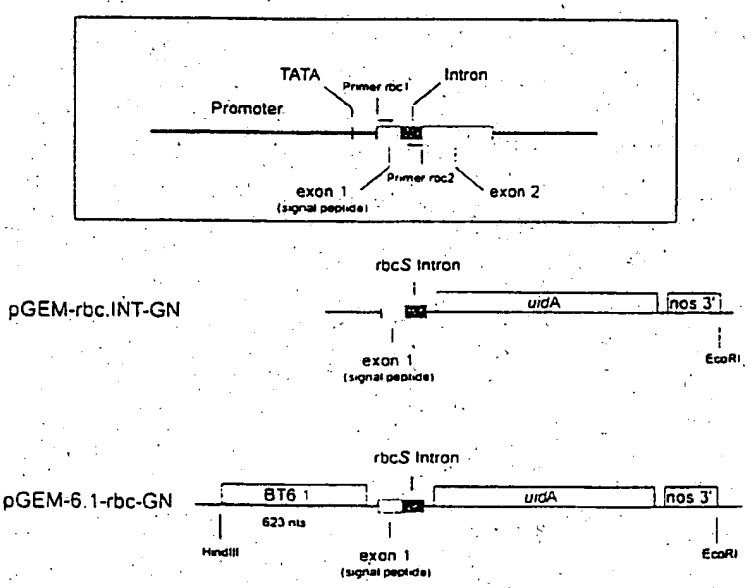

TATA
Primer rbc1
Intron
Promoter
Primer rbc2
exon 1
(signal peptide)
exon 2
pGEM-rbc.INT-GN
rbcS Intron
uidA
nos 3'
exon 1
(signal peptide)
EcoRI
pGEM-6.1-rbc-GN
rbcS Intron
BT6.1
623 nts
uidA
nos 3'
HindIII
exon 1
(signal peptide)
EcoRI

Figure 14

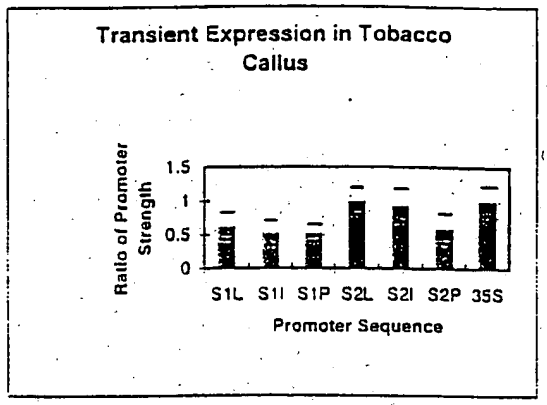

Transient Expression in Tobacco Callus
Ratio of Promoter Strength
1.5
1
0.5
0
S1L S1I S1P S2L S2I S2P 35S
Promoter Sequence

Figure 15

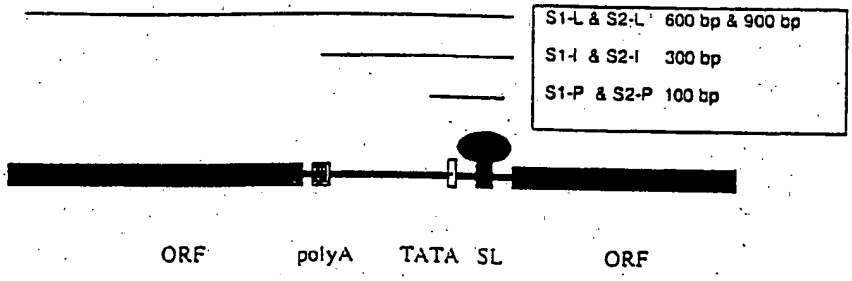

S1-L & S2-L 600 bp & 900 bp
S1-I & S2-I 300 bp
S1-P & S2-P 100 bp
ORF
polyA
TATA
SL
ORF

Figure 16a

Figure 16b

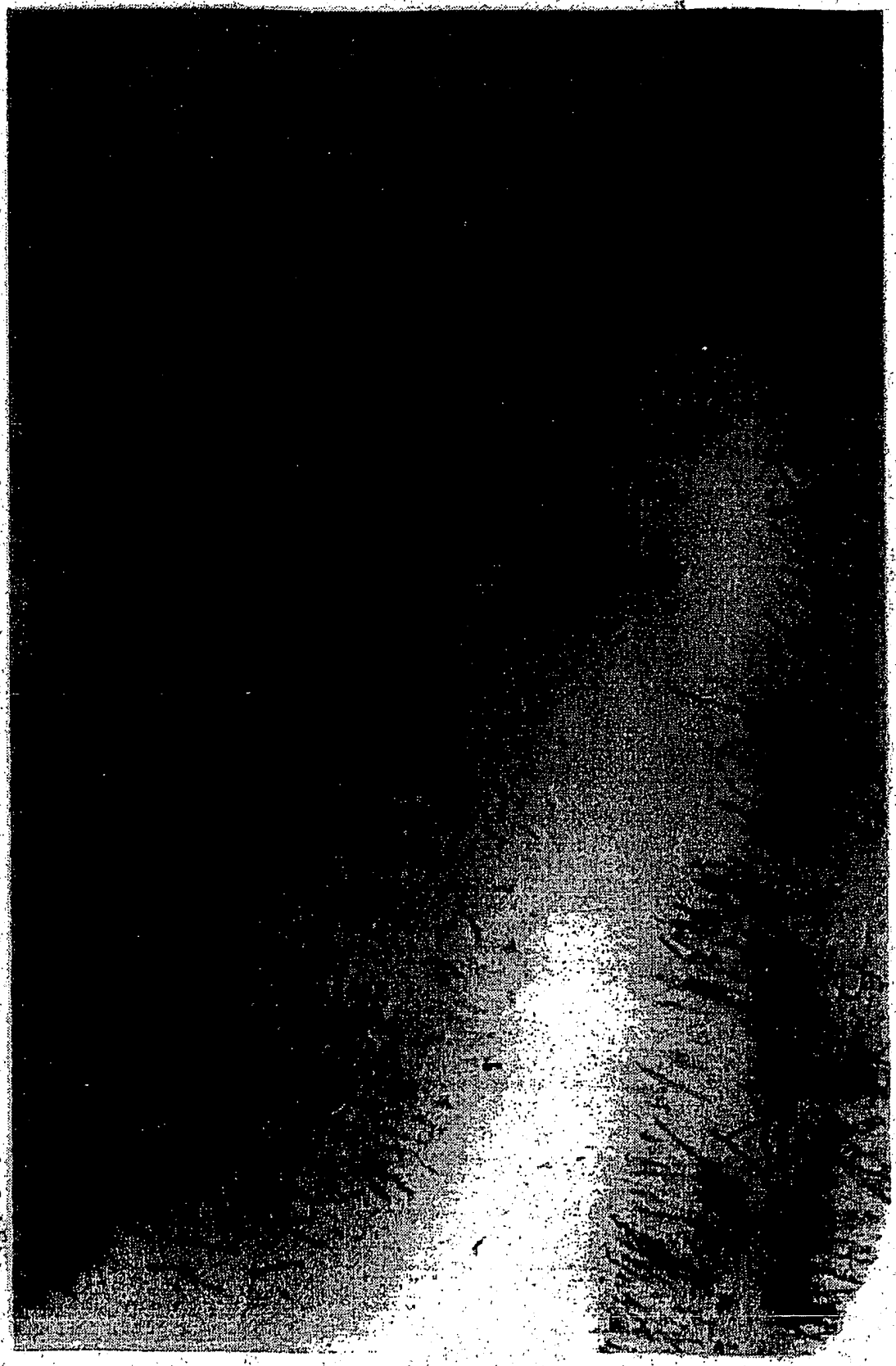


Figure 17

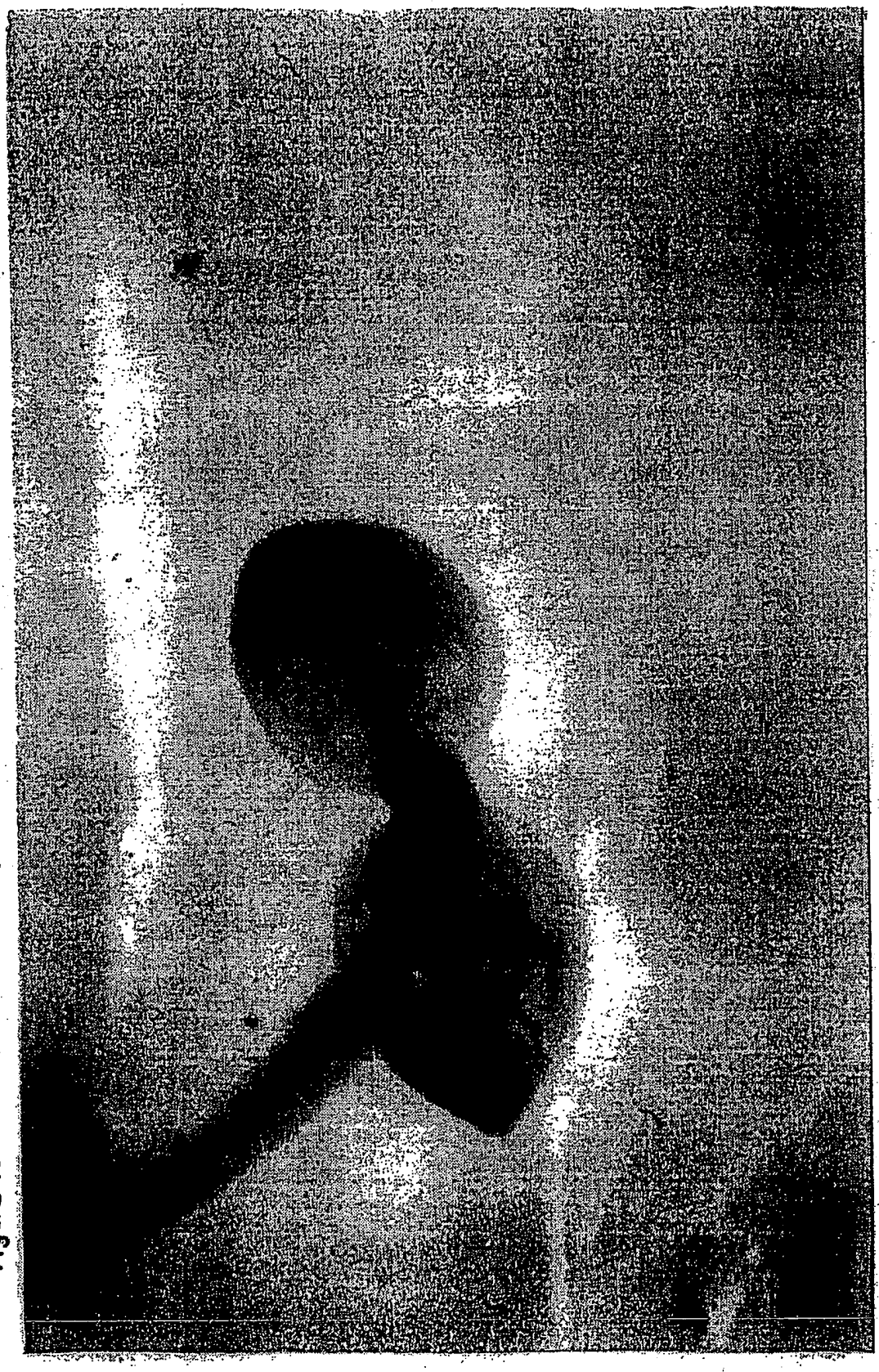


Figure 18

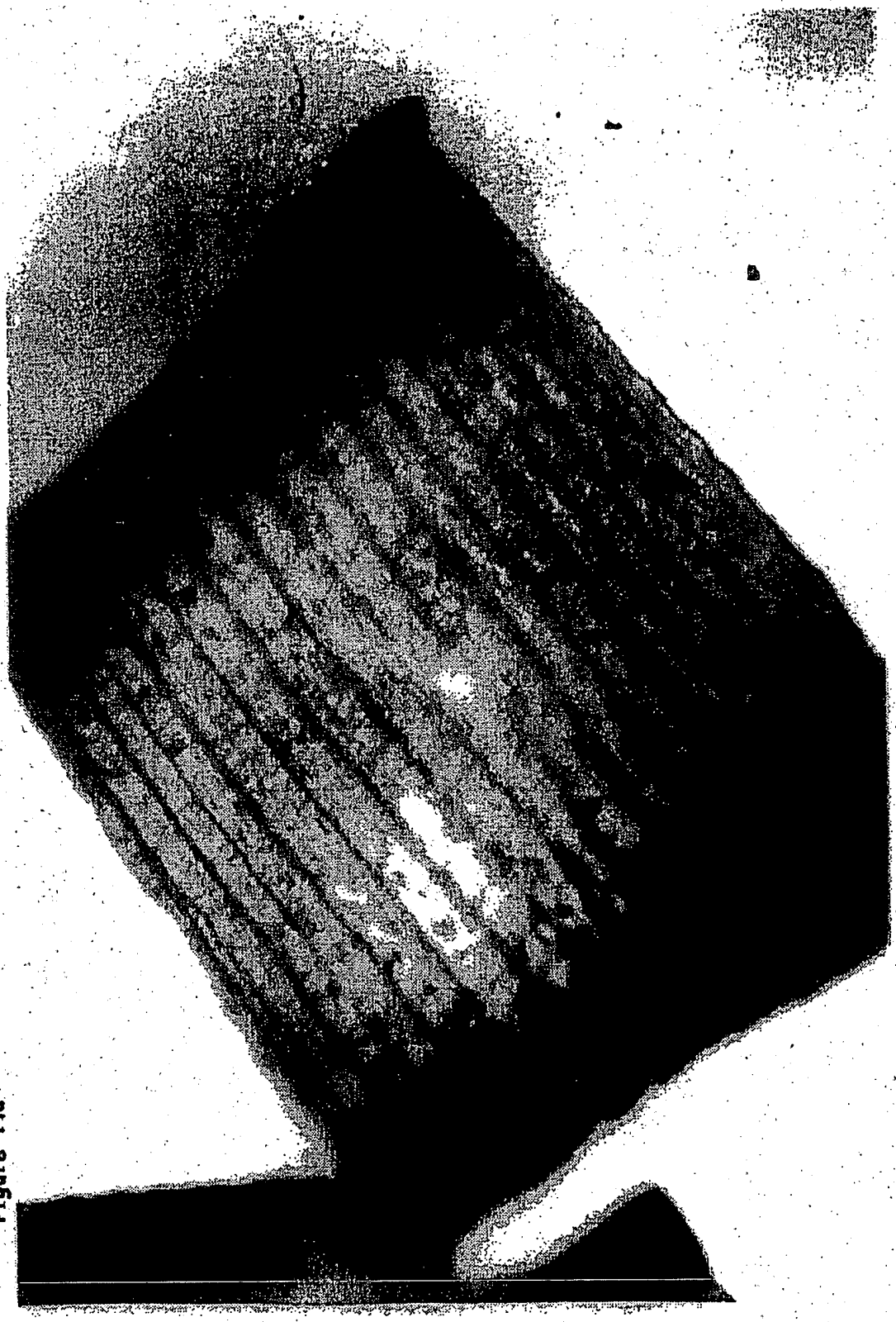


Figure 19a

Figure 19b

Figure 20

Figure 21a

Figure 21b

Figure 22 23/25

S1 Promoter

GGTCTATGGTCCGAATGGTAATGAGGGGAAATCAACATATGCGAAGTCA
TTAATGAAGAAGGACTGGTTCTACACCAGAGGTGGGAAGAAGGAGAACA
TACTGTTCTCTTACGTGGACGAAGGATCTGAGAAGCATATTGTATTTGAT
ATTCCTCGCTGTAATCAGGATTATT
TAAATTATGATGTTATAGAGGCATTAAAGGATAGGGTGATAGAGAGTACT
AAATATAAACCTATTAAGTTAGTTGAATTGATTAATATACATGTAATTGT
CATGGCTAATTTCATGCCAGAATTCTGTAAAATC*TCCGAAGATAGAATAA*
AGATTATTTATTGTTAAAAAGGAAATTATATTATGCACTATGACAATCGT
ACGCTATGACAAAAGGGGACCACAAAGACTCGGGGGTTGATTGCGACATC
CTAACGATTAAGGGCCGCAGGCCCGTCAAGATGGAATGAACGGTCAGATT
TGATTGCTTAGCCACGAAGGAACAACTTTAAGTTCCTCGCCCCTATATAT
AGTTTTGCCGAGGAAGTGGCCTAGTATTACCCACTTCCTCGCCCTTCTTCCTCGCC
CCTACGTCATCAGT

Figure 23

S2 Promoter

GGCTCACTGGGAGAGGGCGAGAGGAACTGATGAACAGAATCGCGGATAC
TGTTCGAAGGAAACCCTAGTTCTTGAACTGGGTACTCCGGTGGTCCCTG
GTTCGAAGAAGCGCAAGCTTCTCGAGAGATTCAGAGAGAGCCCTGAAGA
ATTGAAGATGGAGGATCCATCCAAGTATCGC
AGATGCTTGGCAGTGGAATCGTTGAACAATGCTAGGAAAAATTCTGAAT
GGGTTCATGAACTAAGAGAATGGCAAAATAAATTAATTCAACACATCGAA
GGTGTTCCTGATGATCGAAGTATCATCTGGGTATACGGTCCCAACGGAG
GCGAAGGAAAGTCAACCTTCGCAAGATATCTATCATTAAAACCTGGATGG
GGATATATCAACGGTGGAAAGACGTCGGATATGATGCACATCATAACGA
TGGATCCTGATAATCATTGGATTATTGATATCCCCAGAAGTCATTCAGAT
TATCTGAATTATGGCGTTATAGAACAAATTAAGAATAGAGTTTTAATAAA
TACAAAATACGAACCATGTGTGATTAGAAAAGATGGACAAAATGTCCATG
TAATTGTTATGGCAAATGTGTTGCCTGATTATTGTAAAATTT*CAGAAGATA*
GAATAAAAATAATTAATTGTTGAGAAAGGAAACTTTATCCGCAAGCAATCAA
AAAAGCACGTGGACCCCACACGGTAGCTTGCAGAACACGCTATCATTAAA
TGCATCAAAAAATCATTATAATTAATAAATCTCTTATTGGGCCGCAGGCC
CATTAAGAATCGGCCCTTAATGGGCCGACCTCCTCGCCCTATATA
AGGAGGAGCGCCTAGTATTACCGCTCCTCCTCGCCTTTCCTCCTCGCCCC
TGACGTCATCATT

FIGURE 24

Promoter	Promoter Strength	Tissue Specificty	
	(0-10:weak:strong)	Tobacco	Banana
S1L	5	Lvt,Rvt,Rm,Rh	Lvt,Rvt,Rm,Rh
S1I	1	Rvt	-
S1P	1	Rvt	-
S2I	3	Rm,Rh,Pl	Rm
S2P	0	-	-
S1Iubi	10	-	Lvt,Rc
S2Iubi	10	-	Lvt,Rc

SEQUENCE LISTING

<110> QUEENSLAND UNIVERSITY OF TECHNOLOGY

<120> DNA PROMOTER SEQUENCES DERIVED FROM BANANA BUNCHY TOP VIRUS

<130> QUE00547.05I

<140> PCT/AU98/00786
<141> 1998-09-21

<150> AU PO9399
<151> 1997-09-19

<150> AU PP4423
<151> 1998-06-30

<160> 20

<170> PatentIn Ver. 2.0

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<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: PRIMER 1

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<210> 2
<211> 22
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<210> 3
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<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: PRIMER 3

<400> 3
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<210> 4
<211> 18
<212> DNA
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<220>
<223> Description of Artificial Sequence: PRIMER 4

<400> 4
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<210> 5
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<220>
<223> Description of Artificial Sequence: PRIMER rbc1

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<210> 6
<211> 24
<212> DNA
<213> Artificial Sequence

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<210> 7
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<223> Description of Artificial Sequence: PRIMER BT6.ter

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<210> 9
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<210> 10
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<400> 10
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<210> 11
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<210> 12
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<400> 12
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<210> 13
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<210> 14
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<212> DNA
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<400> 14
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<210> 15
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<220>
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<400> 16
gcgtcgacgg ctcactggga ga 22

<210> 17
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<212> DNA
<213> Banana bunchy top virus

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taagtataat gtcacgtgcc gaattaaaag gttgcttcgc cacgaagaaa cctaatttga 540
ggttgcgtat tcaatacgct accgaatatc tattaatatg tgagtctctg ccgaaaaaaa 600
tcagagcgaa agcggaaggc agaagcgatg 630

<210> 18
<211> 203
<212> DNA
<213> Banana bunchy top virus

<400> 18
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ttaataaaac gtatatttgg gaaattgata gttgtataaa acatacaaca cactatgaaa 180
tacaagacgc tatgacaaat gta 203

<210> 19
<211> 593
<212> DNA
<213> Banana bunchy top virus

<400> 19
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gttagttgaa ttgattaata tacatgtaat tgtcatggct aatttcatgc cagaattctg 300
taaaatctcc gaagatagaa taaagattat ttattgttaa aaaggaaatt atattatgca 360

ctatgacaat cgtacgctat gacaaaaggg gaccacaaag actcgggggt tgattgcgac 420
atcctaacga ttaagggccg caggcccgtc aagatggaat gaacggtcag atttgattgc 480
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<210> 20
<211> 886
<212> DNA
<213> Banana bunchy top virus

<400> 20
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cgagagattc agagagagcc ctgaagaatt gaagatggag gatccatcca agtatcgcag 180
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ttatccgcaa gcaatcaaaa aagcacgtgg accccacacg gtagcttgca gaacacgcta 720
tcattaaatg catcaaaaaa tcattataat taataaatct cttattgggc cgcaggccca 780
ttaagaatcg gcccttaatg ggccgacctc ctcgccctat ataaggagga gcgcctagta 840
ttaccgctcc tcctcgcctt tcctcctcgc ccctgacgtc atcatt 886

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 98/00786

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[6]: C12N 15/11

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
EMBL, Gen Bank, Biosys, Medline

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Chem Abs, Biosys, Medline: (Banana()Bunchy()Top()Virus or BBTV) and (Promoter or Untranslat: or Non coding or noncoding)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	J. Gen. Virology, v.76, 1885, pp 1471-1482, BURNS T.M. *et al*, "The genome organization of banana bunchy top virus: analysis of six ssDNA components" Abstract, Page 1479	1-4
A	Virology, v.198, 1994, pp 645-652, YEH H.H. *et al*, "Genome characterization and identification of viral-associated dsDNA component of banana bunchy top virus"	
A	EMBL data accession no. U12586, BB12586, 18 February 1995, WU R.Y. & YOU L.R.	

[X] Further documents are listed in the continuation of Box C

[ ] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 October 1998	- 2 NOV 1998
Name and mailing address of the ISA/AU AUSTRALIAN INDUSTRIAL PROPERTY ORGANISATION PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No.: (06) 285 3929	Authorized officer **BARRY SPENCER** Telephone No.: (06) 283 2284

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 98/00786

C (Continuation) **DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EMBL data accession no. U12587, BB12587, 18 February 1995, WU R.Y. & YOU L.R.	
A	EMBL data accession no. L32166, LEBYTV1, 11 August 1994, WU R.Y., YOU L.R. & SOONG T.S.	
A	EMBL data accession no. L32167, LEBYTV2, 11 August 1994, WU R.Y., YOU L.R. & SOONG T.S.	